Solid-phase protein PEGylation: Achieving mono-PEGylation through molecular tethering

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Abstract

Protein PEGylation (covalent attachment of poly(ethylene glycol) or PEG to proteins) is an excellent example of a drug delivery system that improves pharmacokinetics and pharmacodynamic properties of therapeutics. However, although PEGylation is clinically proven and attracts both scientific and commercial interest, the technique is associated with many process constraints, in particular related to controlling the number of conjugated PEG chains. A novel, solid-phase PEGylation methodology was attempted to overcome the drawbacks of the commonly used solution-phase methods for preparing PEGylated products. The solid-phase PEGylation methodology involved conjugating protein onto a tethered PEG derivative attached onto a solid matrix, followed by hydrolytic cleavage of the PEG chain from the solid matrix under mild conditions to yield PEGylated protein in free solution.

PEGs with molecular weights (MWs) 2000 and 4000 Da were used and a heterobifunctional PEG derivative, α-(β-alanine)-ω-carboxy PEG, with a cleavable β-alanine ester terminal was prepared for surface grafting and protein conjugation. The amine terminal of this PEG derivative was used for grafting PEG onto carboxy functionalized hydrophilic Sephadex and hydrophobic polystyrene derivatives. The free carboxyl terminal was used for protein conjugation via amine coupling. A kinetic study of PEG-surface grafting was performed to understand the influence of a number of parameters on the PEG surface concentration and its conformation, including temperature, reaction time, nature of the matrix, solvent and base, and MW of PEG. PEG grafted matrices were characterized using various surface characterization tools including Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS).

Higher PEG grafting was observed with polystyrene matrices (up to 0.3 mmol/g) than either of the Sephadex derivatives (less than 0.15 mmol/g) using both molecular weights. Detailed surface characterization using XPS studies showed a layer thickness of 11.87 nm was achieved with polystyrene matrices using 4000 Da PEG derivatives after a grafting period of 72 hours at 40°C, indicating the presence of brush conformations for the grafted PEGs. In contrast, mushroom conformations were observed for PEG molecules grafted on both carboxymethyl and carboxypentyl Sephadex derivatives after the same reaction period, with a layer thickness of 2.62 nm and 4.14 nm respectively.

Optimized PEG grafting and hydrolysis conditions were developed for solid-phase protein PEGylation using Cytochrome c as a model protein. The presence of PEGylated species were detected by size exclusion chromatography (SEC) from Sephadex derivatives but were absent when using polystyrene matrices. Both Sephadex derivatives gave mainly multi-PEGylated species with poor yields, in place of the expected mono-PEGylated products. A solution-phase PEGylation using the same PEG derivatives was performed successfully and various PEGylated species were identified and characterized using SEC and gel electrophoresis, based on their viscosity radius.

An examination of the surface characteristics of the PEG-grafted was carried out by XPS, showing that protein conjugation was greatly influenced by surface force interactions, which depended on the PEG grafting densities and the nature of the solid matrices. Finally, fluorescent images obtained using confocal microscope with fluorescein isothiocyanate labelled Cytochrome c provided supporting evidence regarding the factors that constrained the solid-phase PEGylation process.

Acknowledgments

First of all I wish to thank my senior supervisor, Professor Conan J. Fee, for his invaluable guidance, discussions and kind encouragements throughout my studies. His great understanding and supportive nature were my main source of encouragement, which made me confident and motivated. I cherish every moment of our association in the past four years!

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And finally, I would like to dedicate this thesis to my own Sree, who has been always with me during the toughest times, to support my dreams.

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Abbreviations

Boc	tert-butoxycarbonyl
CLSM	confocal laser scanning microscopy
CM	carboxy methyl
COA	certificate of analysis
CP	carboxy pentyl
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	dicyclohexyl carbodiimide
DCM	dichloromethane
DHU	dicyclohexyl urea
DI water	deionized water
DIEA	N,N-diisopropylethylamine
DMAP	dimethyl aminopyridine
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
EDC	1-ethyl-3-(3-dimethylamino)propyl carbodiimide
eq.	equivalent (molar)
FDA	Food and Drug Administration (US)
FITC	fluorescein isothiocyanate
FTIR	fourier transform infrared spectroscopy
HCl	hydrochloric acid
H_2SO_4	Sulphuric acid
IPA	isopropyl alcohol
KBr	potassium bromide
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
KOH	potassium hydroxide
LiBr	lithium bromide
MBHA	4-methylbenzhydrylamine

MCA	monochlroroacetic acid
MeOH	methanol
$MgCl_2$	magnesium chloride
mPEG	methoxy PEG
MO	molecular orbitals
MW	molecular weight
N_2	nitrogen
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NHS	N-hydroxysuccinimide
NMM	N-methylmorpholine
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
$Pd(PPH_3)_4$	tetrakis(triphenylphosphine) palladium (0)
Pd/C	palladium adsorbed on charcoal
PEG	poly (ethylene glycol)
PS	polystyrene
R_F	Flory radius
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SEM	scanning electron microscopy
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TsCl	p-toluene sulfonyl chloride
UV	ultra violet
vol.	volume

XPS	X-ray photoelectron spectroscopy

1 Introduction

1.1 Background and significance

Innovative drug delivery technologies are key components of drug development, with commercial as well as intellectual values. PEGylation, the process of attaching poly(ethylene glycol) (PEG) to a molecule, is an excellent example of such a delivery system characterized by its remarkable improvement in pharmacokinetics and the pharmacodynamic values of therapeutics, especially for proteins, peptides and even small molecule pharmaceuticals. PEG, a non-toxic biocompatible polymer, can be attached to a therapeutic molecule through a permanent or reversible linkage and will provide a protective covering to the molecule from proteolytic degradation and renal clearance. Consequently, the method has resulted in the development of a number of drugs with enhanced blood circulatory life and lower administration frequency than a non-PEGylated drug (Figure 1-1).

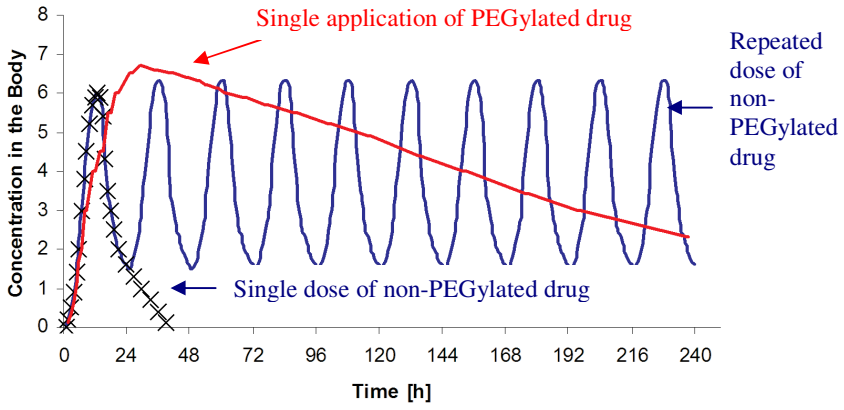


Figure 1-1: Pharmacokinetics of PEGylated drugs (Bruckdorfer 2008)

Although there are a number of FDA-approved PEGylated drugs already in the market and many under clinical trials, this technology is accompanied by various processing challenges such as difficulty with controlling reaction selectivity, the extent of PEG conjugation, positional isomerism, low efficiency in the separation of various PEGylated products and unreacted starting materials, and often low yields of the desired PEGylated product (Fee and Van Alstine 2006). Consequently, current processes commonly result in a mixture of mono, di and oligo-PEGylated products (Fee 2003), requiring complex and multistep downstream purification steps to isolate the required product. The very high cost of therapeutic proteins and the high costs associated with the above PEGylation processing and separation issues results in extremely expensive products.

Consequently, there is significant scientific interest and importance in developing a novel reaction engineering design to improve the efficiency and yield of the existing PEGylation process. Paramount requirements include minimizing the variation between batches and significantly improving the yield and selectivity of the PEGylation process. This should result in lower production costs and improved availability of the resulting therapeutics. Also this technology will enable the pharmaceutical and biotech industries to manufacture drugs consistently with specific characteristics and with minimal variations between batches to meet stringent regulatory requirements.

1.2 Solid-phase protein PEGylation – an innovative approach

Considering the various limitations and process variations associated with the existing technology, a novel and innovative solid-phase PEGylation design was proposed to minimize the variations between the

batches and to increase the specificity and yield of the process. The concept involves the use of a tethered PEG molecule attached to a modified solid matrix through a cleavable linker. The free end terminal of the PEG molecule would be modified with a functional group capable of selective conjugation with a particular functional moiety on the protein surface. Taking advantage of surface force interactions between the PEG grafted matrix and protein, an accumulation of protein was anticipated near the terminal end of tethered PEG chains and thereby expected to result in conjugation. Finally, the PEGylated protein would be separated from the matrix by cleaving off the labile linker using mild hydrolytic conditions.

1.3 Project objectives

The main goal of the current project was to develop a novel solid-phase PEGylation strategy through the following objectives:

- The preparation and evaluation of a suitable heterobifunctional PEG derivative capable of tethering a conjugated protein onto a solid-phase resin through a cleavable linker.
- The optimization of the PEG grafting conditions to obtain a preferred conformation which enables successful conjugation of protein onto the matrix.
- The establishment of suitable hydrolysis conditions to release the PEGylated product from the resin after protein conjugation without denaturing the protein.
- An exploration of the adaptability of the solid-phase PEGylation technique using various solid matrices

- The systematic study, using suitable analytical tools, of various conformational behaviours of surface grafted PEG molecules and correlating these with theoretical predictions.
- The study of the various surface force interactions exerted between the protein and PEG grafted matrices during conjugation and their influence on the performance of the solid-phase PEGylation system.

1.4 Thesis organization

Based on the objectives, remainder of this thesis is organized as follows:

Chapter 2: Following a brief overview of the development of protein PEGylation in terms of the improvement in conjugation chemistry and various process modifications, the requirement for an improved methodology to overcome the present drawbacks is described. The concept of the novel methodology is presented with a concise description of its various components and finally a theoretical prediction of the performance of the technique is included.

Chapter 3: This chapter describes the various synthetic strategies for preparing heterobifunctional PEG derivatives and their solution-phase PEGylation with the model protein.

Chapter 4: The optimization of PEG grafting, hydrolysis and solid-phase PEGylation form the main focus of this chapter. The preparation of carboxy tethered matrices and the influence of various reaction parameters on the PEG grafting are also included.

Chapter 5: The surface characterization of PEG grafted matrices using various analytical tools is described. A prediction concerning the protein interaction with PEG grafted matrices and their influence on the performance of the developed solid-phase system is included.

Chapter 6: In this chapter a detailed analysis of the overall results is included based on XPS data and various surface force interactions.

Chapter 7: Conclusion and recommendations for future studies are summarized in this chapter.

Appendices A and B: A brief summary of other attempted synthetic approaches and XPS supplementary results are given in these appendices.

2 Literature review

2.1 Introduction

This chapter mainly describes the development of a versatile and clinically proven polymeric drug delivery system known as protein PEGylation, followed by various process considerations and constraints of the existing method, and finally the strategy behind the development of an improved technique. A detailed insight into various conjugation chemistries as well as process variations is also included. The need is ascertained for a highly selective and specific methodology to improve on the existing ones, considering the pharmacological and commercial importance of the PEGylated drugs. An innovative solid-phase PEGylation system is described, that has an emphasis on reducing the variation between the batches and the heterogeneity of the PEGylated products. A prediction of the performance of the proposed technique, based on theoretical calculations, is also included in this chapter.

2.2 Bioconjugation and polymer therapeutics

The rapid growth in biotechnology and molecular biology during the last two decades has contributed to a substantial increase in the number of biotechnological and biological products such as proteins, peptides, hormones and enzymes for pharmaceutical applications (Blohm et al. 1988; Lubiniecki 1997). This pharmaceutical significance of these biomolecules can be mainly attributed to their high specificity, rapid onset of action and requirement for relatively smaller dosage forms compared to conventional synthetic molecules (Wu-Pong and Rojanasakul 1999). Unfortunately, most of these biomolecules are

characterized by their short circulating lives, low stability due to proteolytic and enzymatic degradations *in vivo* and fast clearance of small molecules from the body via glomerular (kidney) filtration (Nucci et al. 1991; Pasut and Veronese 2007).

In order to overcome these drawbacks, these biomolecules can be protected by converting them into a macromolecular complex through a covalent binding process with another molecule known as bioconjugation (Veronese and Morpurgo 1999). Many polymers having biological and synthetic origins are proved and extensively used to protect biomolecules, to overcome the above-mentioned drawbacks. The resulting "polymer bioconjugates" (Figure 2-1) are characterized by some additional favourable properties such as reduced immunogenicity, decreased antibody recognition, increased *in vivo* residence time, increased drug targeting specificity and bioavailability, and improved pharmacokinetics (Veronese and Morpurgo 1999; Duncan 2003; Khandare and Minko 2006; Lutz and Borner 2008; Gaspar and Duncan 2009).

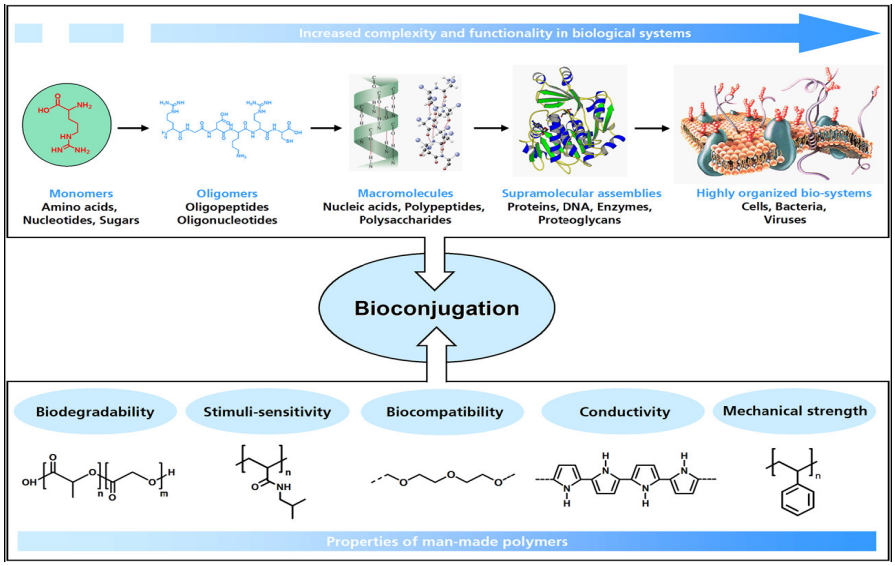


Figure 2-1: Concept of polymer bioconjugation (Lutz and Borner 2008)

Taking advantage of these improvements, the concept of polymer bioconjugation is extended to small, high-value prodrugs and leads into a new era of polymeric drug delivery systems and polymer therapeutics (Khandare and Minko 2006). Commonly used polymers in drug delivery applications include poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA) (Schoemaker et al. 2002; Seymour et al. 2002; Tao et al. 2009), poly(oligoethylene glycol methyl ether methacrylate) (POEGMA) (Lele et al. 2005; Popescu et al. 2005; Lutz et al. 2007), poly(D,L-lactic-co-glycolic acid) (PLGA) (Nam and Park 1999), poly(glutamic acid) (PGA) (Bhatt et al. 2001; Zou et al. 2001), poly(N-isopropyl acrylamide) (PNIPAM) (Chen and Hoffman 1993; Takei et al. 1993; Gil and Hudson 2004), poly(N,N'-diethyl acrylamide) (PDEAM) (Gil and Hudson 2004), polystyrene (Velonia et al. 2002) and poly(ethylene glycol) (PEG).

2.3 PEG – the polymer of choice

The specific characteristic properties of PEG such as biocompatibility, lack of immunogenicity, antigenicity and toxicity, solubility in water and other organic solvents, ready clearance from the body and high mobility in solution make this the polymer of choice for bioconjugation (Harris 1992; Roberts et al. 2002; Pasut and Veronese 2006). The conjugation of a biomolecule with PEG will result in the modification of its physiochemical properties, particularly size, and increase the systemic retention of the therapeutic agent in the body; it will also enable the moiety to cross the cell membranes by endocytosis to reach particular intracellular targets (Khandare and Minko 2006). Moreover, PEG is one among the small number of synthetic polymers approved by the US FDA for internal administration (Bhattarai et al. 2005). Detailed descriptions of the various properties of PEGs are given in Section 2.6.4.

2.4 Protein PEGylation

In the late 1970s, Prof. Frank Davis and his colleagues covalently linked methoxy-PEG (mPEG) to bovine serum albumin (Abuchowski et al. 1977a) and bovine liver catalase (Abuchowski et al. 1977b), using cyanuric chloride as an activating agent. Their studies showed that "hanging a bit of PEG onto a protein" markedly improved the overall properties and stability of the protein (Davis 2002; Katsnelson 2006). This technique is now well established and is known as "PEGylation". The applications of PEGylation can be extended to peptides, enzymes, antibody fragments, nucleotides and even small organic molecules to meet the challenges of improving the safety and efficiency of many therapeutics (Matsushima et al. 1996; Riley and Riggs-Sauthier 2008).

2.4.1 Pharmaceutical significance of protein PEGylation

PEGylation can impart several significant and distinct pharmacological advantages over the unmodified form, including improved drug solubility, reduced dosage frequency, toxicity and rate of kidney clearance, extended circulating life, increased drug stability, enhanced protection from proteolytic degradation, decreased immunogenicity and antigenicity, and minimal loss of biological activity (Nucci et al. 1991; Bailon and Berthold 1998; Kozlowski and Harris 2001; Veronese and Pasut 2005).

The reduced kidney clearance of PEGylated proteins can be attributed to an apparent shielding of protein surface charges as well as an increased hydrodynamic volume of the conjugated product (Caliceti and Veronese 2003) due to the ability of PEG molecules to coordinate with two to three water molecules per monomer unit (Antonsen and Hoffman 1992)

(Section 2.6.4.2.1). Furthermore, these hydrated polymer chains provide a protecting mask for the protein epitope, thereby resulting in a decreased immunogenicity and antigenicity, which is found to decrease with an increase in the number and molecular weights of the attached PEGs. Moreover, the masking of these recognition sites and charges reduces the phagocytic uptake by parenchimal cells, by preventing the opsonisation processes and increasing their residence period in systemic circulation (Caliceti and Veronese 2003; Hamidi et al. 2006).

In addition to these pharmacological advantages, PEGylation can substantially alter many physicochemical properties of the parent protein, including changes in conformation, electrostatic binding, and hydrophobicity (Bailon and Berthold 1998). PEGylation significantly influences the elimination pathway of the molecule, by shifting from a renal to a hepatic pathway with an increase in the number and molecular weights of the attached PEG molecule. The tissue-organ distribution profile of the molecule is also greatly influenced by PEGylation, as PEGylated proteins medicated by an intravenous administration preferably follow a peripheral distribution (Caliceti and Veronese 2003; Hamidi et al. 2006). The pharmaceutical value of PEGylation is now well accepted, with many FDA approved drugs already launched in the market and many in clinical trials. A summary of PEGylated drugs marketed and in various developmental stages is given in Table 2-1 and 2-2.

Table 2-1: PEGylated drugs on the market (Storz and Lu 2008)

Name	Company (Year)	Indication	Parent molecule	PEG
Adagen®	Enzon (1990)	Severe combined immunodeficiency disease (SCID)	adenosine deaminase	11-17 5 kDa linear
Oncasper®	Enzon (1994)	Lymphoblastic leukemia	asparaginase	11-17 5 kDa linear
PEG-Intron®	Schering- Plough / Enzon (2001)	Hepatitis C	Interferon-α-2b	12 kDa linear
Neulasta®	Amgen (2002)	Neutropenia during chemotherapy	G-CSF	20 kDa linear
Pegasys®	Roche (2002)	Hepatitis C	Interferon-α-2a	40 kDa branched
Somavert®	Pfizer (2003)	Acromegaly	growth hormone receptor	4-5 5 kDa linear
Macugen®	Eyetech (2004)	Age-related macular degeneration	anti-VEGF 24-mer oligonucleotide	40 kDa branched
Mircera®	Roche / Nektar (2007)	Anemia in chronic kidney disease (CKD)	B-EPO	30 kDa linear
Cimzla®	UCB / Nektar (2008)	Crohn's disease	anti-TNF-αhAB fragment	two 2 kDa branched

Table 2-2: PEGylated drugs in development (Storz and Lu 2008)

Name	Company	Indication (Approximate clinical status)	Parent molecule	PEG
Hematide™	Affymax/ Takeda/ Nektar	Anemia in CKD and cancer (Phase III)	12-18 amino acid peptide dimer	two 20 kDa linear
PEG-hGRF	Serono	Growth hormone deficiency (Phase I)	29 amino acid peptide	Mixture of two mono 5 kDa PEGylated isomers
3DP-3534	J&J	Thrombocytopen-ia (Preclinical)	14 amino acid peptide dimer	two 20 kDa linear
PEG-thymalfasin	SciClone / Nektar	HBV, HCV, melanoma, liver cancer (Preclinical)	Zadaxin (thymosin-α1, 28 amino acid peptide)	--
PEG-glucagon-like peptide-1	Bayer	Diabetes (Preclinical)	30-35 amino acid peptide	22 or 43 kDa
PEG-vapreotide	Debiopha-rm	Various cancers (Preclinical)	synthetic octapeptide somatostatin analog	5 or 2 kDa linear
PEG-c(RGDyK)-peptide	University of California / UIPS	Angiogenesis inhibitors (Preclinical)	Cyclic pentapeptid-es	various

2.4.2 The chemistry of protein conjugation

The development of PEGylation technology is signified with a marked improvement in conjugation strategy, from non-specific random conjugations in the early stages, known as the "first generation PEGylation," to the recent site-specific conjugation methods known as the "second generation PEGylation" (Pasut and Veronese 2006). This increase in specificity of PEGylation can be mainly credited to the availability of more specific functionalization of PEG molecules capable of reacting to a particular functional moiety present with the protein under conjugation. This results in controlled, well-defined conjugated products with an improvement in product profile over those obtained from non-specific random conjugations.

2.4.2.1 Amine conjugation

Owing to the availability of a number of accessible primary amino groups on the surface of a protein, conjugation through this functional group is the most extensively used method in protein PEGylation. Lysine, ornithine and N-terminal amino groups are the most commonly explored anchoring sites for PEG conjugation (Bruckdorfer 2008). Early stage developments of PEGylation were mainly based on this chemistry, targeting N-terminal amino groups of lysine. The very first reactions reported by Davis and his colleagues involved the reaction of cyanuric chloride activated PEG with the primary amine groups present on bovine serum albumin (Abuchowski et al. 1977a) and bovine liver catalase (Abuchowski et al. 1977b), through alkylation of their respective amine terminals (Figure 2-2). Thereafter, PEG-tresylate was also developed for protein conjugation through alkylation (Gais and Ruppert 1995) but all these reactions resulted in non-specific multiple conjugated products.

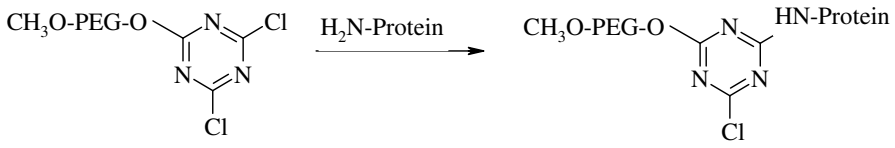


Figure 2-2: Protein conjugation using cyanuric chloride activated PEG

A greater specificity and selectivity in N-alkyl conjugation strategy was developed after the introduction of PEG aldehyde derivatives, particularly mPEG-propionaldehyde (Harris and Sedaghat-Herati 1993), capable of forming a stable secondary amine linkage with amino groups through reductive amination using sodium cyanoborohydride (Borch et al. 1971; Baxter and Reitz 2002) (Figure 2-3). Because the reactivity of aldehyde groups depends on the nucleophilicity of amine groups, reaction will take place only when the pH of the medium is near or above the pKa of that particular amine terminal. Hence by controlling the pH of the reaction medium, the heterogeneity of the product profile can be greatly reduced (Roberts et al. 2002). This conjugation strategy was adopted for the development of Neulasta® (PEG-G-CSF), where a linear 20 kDa mPEG-aldehyde derivative was selectively attached to an N-terminal methionine residue of filgrastim through reductive alkylation under mild acidic conditions (Kinstler et al. 2002; Veronese and Mero 2008). Another example using this reductive alkylation chemistry is the PEGylation of a recombinant soluble tumor necrosis factor receptor type I (sTNF-RI) from *E. coli*, for use in treating chronic inflammatory diseases (Edwards et al. 2003).

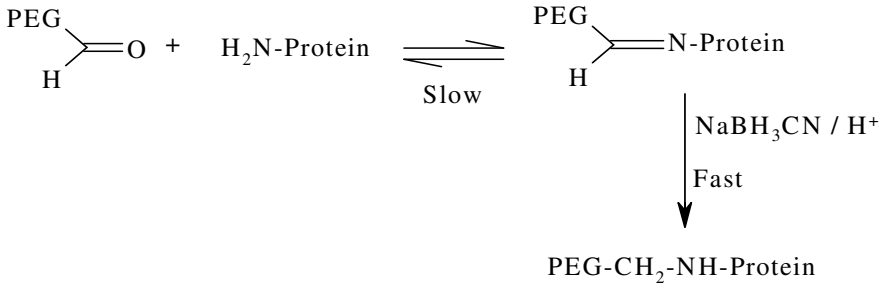


Figure 2-3: Protein conjugation using PEG aldehyde derivative

The acylation of the N-terminal amino acids results in the formation of stable amide (R**CO-N**R'$_2$) and urethane (R-**O-CO-N**R'$_2$) linkages and is extensively used as a conjugation strategy in protein PEGylation, especially with first generation PEGylation chemistries. PEG derivatives activated with succinimidyl succinate (PEG-SS) (Zalipsky and Lee 1992), succinimidyl carbonate (PEG-SC) (Zalipsky et al. 1991), benzotriazole carbonate (PEG-BTC) (Dolence et al. 1996), phenyl carbonate (Veronese et al. 1985; Woodward and Kaufman 1996), carbonylimidazole (Beauchamp et al. 1983), and thiazolidine-2-thione (Greenwald et al. 1996) were used in earlier stages for protein conjugation, following the N-terminal acylation pathway. Adagen® (PEG-adenosine deaminase) and Oncaspar® (PEG-asparaginase), the very first PEGylated products launched in the market, are prepared based on this chemistry by conjugating succinimidyl succinate activated PEGs with their respective proteins (Davis 2003) (Figure 2-4).

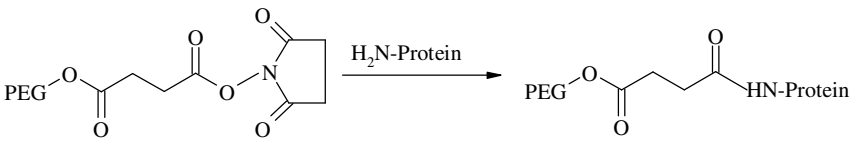


Figure 2-4: Protein conjugation using PEG-SS

PEG-Intron® is prepared by conjugating interferon-α-2b with a single chain 12 kDa PEG-SC via a urethane bond (Figure 2-5), where the PEG is mainly conjugated on to the histidine residue (Wang et al. 2002).

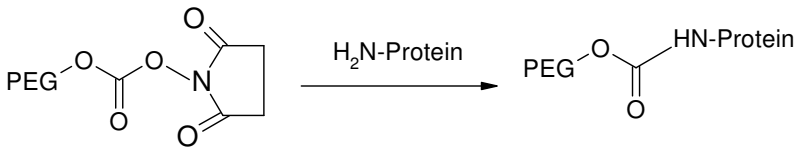


Figure 2-5: Protein conjugation using PEG-SC

The introduction of monosubstituted propionic and butanoic acid PEG derivatives by Harris and Kozlowski (1997) resulted in a substantial improvement in PEG product profile and their subsequent activation using succinimide derivatives contributed a significant improvement in amine conjugation approach. Pegasys® is prepared by mono-PEGylation of interferon-α-2a with an N-hydroxysuccinimide (NHS) activated 40 kDa branched PEG molecule (Bailon et al. 2001) (Figure 2-6).

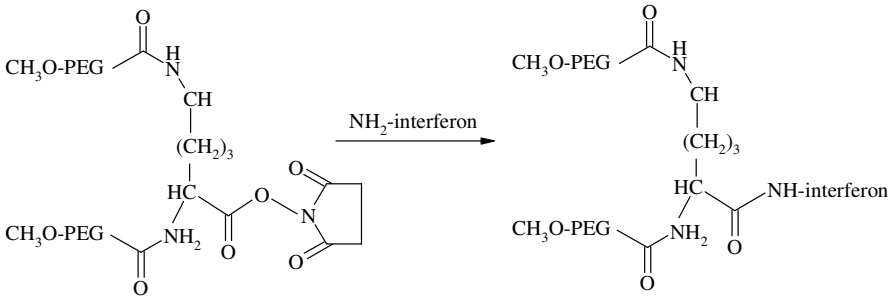


Figure 2-6: Conjugation chemistry for Pegasys®

Another PEGylated drug, Somavert®, is also prepared by conjugating 4 – 5 NHS activated 5 kDa PEG derivatives with a lysine terminal of human growth hormone antagonist (Olson et al. 1997; Parkinson et al. 2003).

Due to the selectivity of NHS active esters towards primary amine terminals, this conjugation technique is now used as one of the most common conjugation strategies for amine coupling (Hermanson 1996). Even though the amine conjugation is widely accepted and clinically proven, the method is associated with some potential disadvantages, including loss of bioactivity due to the inactivation of critical functional groups, and the formation of multi-PEGylated products due to the presence of multiple amino groups available for conjugation (Zappe et al. 2008).

2.4.2.2 Thiol conjugation

Selective thiol conjugation at the natural or genetically engineered unpaired cysteine residues provided another site-specific conjugation methodology for protein PEGylation, with the advantage of minimal loss in bioactivity. Thiol selective derivatives such as PEG-maleimide, vinylsulfone, iodoacetamide, and orthopyridyl disulfide are used for cysteine conjugation through the formation of thioether or disulfide linkages (Roberts et al. 2002). Examples of thiol conjugation using PEG-maleimide include those at the genetically introduced cysteine residue of trichosanthin (TCS) using 5 and 20 kDa (He et al. 1999), antitumor necrosis factor-α-scFv fragment (anti-TNF-α-a scFv) using 5, 20 and 40 kDa (Yang et al. 2003) and recombinant staphylokinase (Sak) using 5, 10 and 20 kDa derivatives (Moreadith and Collen 2003) (Figure 2-7).

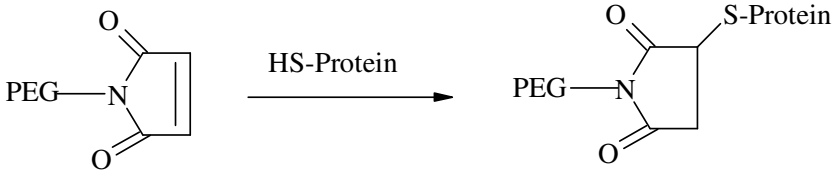
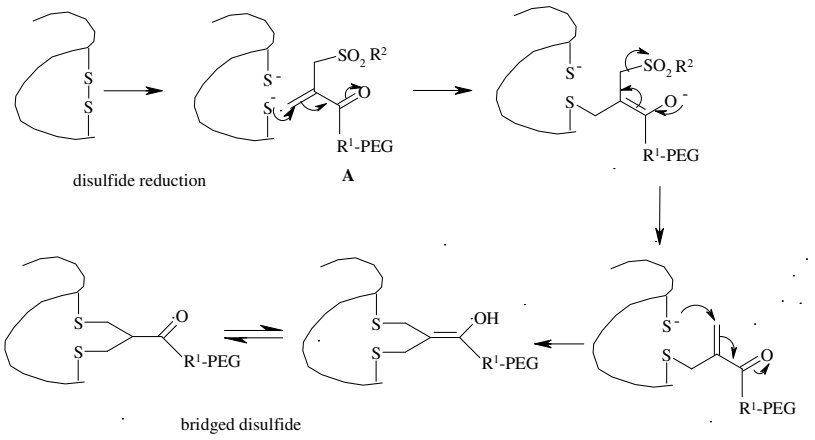


Figure 2-7: Cysteine conjugation using PEG-maleimide

Because of the limited availability of cysteine residues and the chances of protein dimerization resulting from the introduction of genetically engineered cysteines, the use of this strategy is restricted and further modifications in this methodology are preferred. Taking advantage of a higher number of accessible disulfide linkages present with paired cysteines compared to the free unpaired ones, Balan et al. reported a thiol specific bis-alkylation PEGylation, with the two sulphur atoms which were generated by the mild reduction of these disulfide bonds (Balan et al. 2007; Brocchini et al. 2008). A selective reduction of the disulfide bridges was performed using dithiothreitol (DTT) in neutral conditions or tris-(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) in slightly acidic conditions. The reduced protein was then treated with an active ester PEG-monosulfone at an acidic pH (Figure 2-8).



where intermediate **A** represents PEG-monosulfone with the following structure

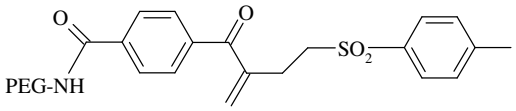
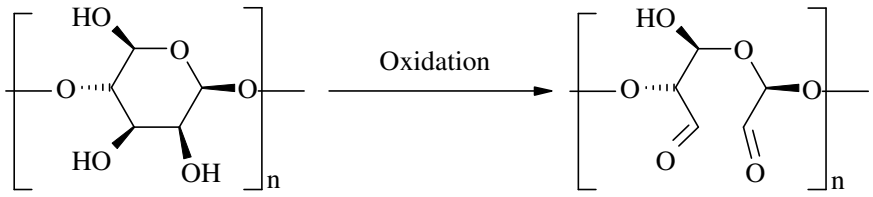


Figure 2-8: Disulfide bridging PEGylation and structure of PEG-monosulfone (Balan et al. 2007)

Steric shielding by the attached PEG molecule against a second molecule approaching the same reduced site, an important requirement for the bridged structure, prevented heterogeneity of the product profile and resulted predominantly in the mono-PEGylated derivative. Also, retaining the protein's tertiary structure and selectivity and reversibility of the thiol conjugation are some of the attractive features of this technique.

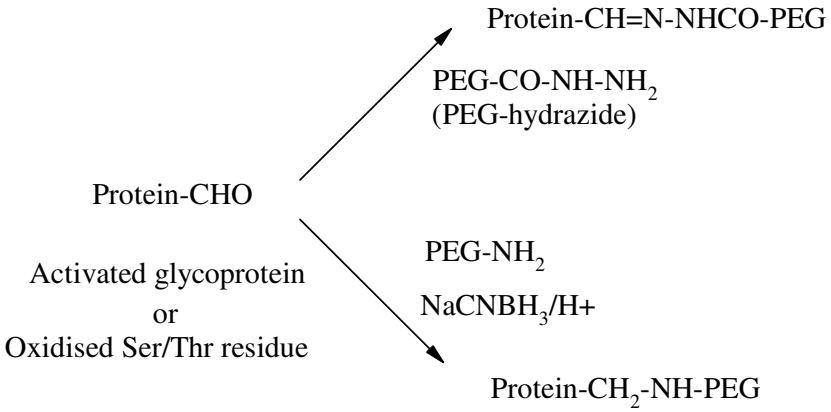
2.4.2.3 Oxidized carbohydrate or N-terminal conjugation

The enzymatic (e.g., glucose oxidase) or chemical (e.g., sodium periodate) oxidation of carbohydrates present with glycoproteins or N-terminal serine or threonine residues generates reactive aldehyde groups, which can be further conjugated with PEG hydrazide or amine derivatives (Hermanson 1996; Zalipsky and Menon-Rudolph 1997; Roberts et al. 2002) (Figure 2-9). Zalipsky and his colleagues used this methodology for PEGylating immunoglobulin G (IgG), which contains nearly 4% carbohydrate; it was first oxidized with periodate and then conjugated with mPEG-hydrazide derivative (Zalipsky and Menon-Rudolph 1997). Following the same chemistry, periodate oxidized Ricin A-chain (RTA), an excellent immunotoxin source, was successfully conjugated with a 5 kDa mPEG-hydrazide (Youn et al. 2005).



Glycoprotein Activated glycoprotein

a) Oxidation of glycoprotein to generate aldehyde groups



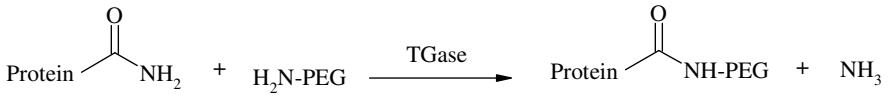
b) PEG conjugation with oxidised protein derivatives

Figure 2-9: Oxidized carbohydrate or N-terminal conjugation

2.4.2.4 Transglutaminase (TGase) mediated enzymatic conjugation

A novel site specific PEGylation methodology targeting glutamine residues was reported by Sato (2002), in which a TGase catalysed acyl transfer reaction between the glutamine (Gln) terminal and PEG primary amino group was used for protein conjugation. Taking advantage of the capability of TGase to recognize a wide variety of alkylamine donors and their high affinity towards long chain aliphatic chains, Sato developed this distinctive strategy by incorporating a short sequence of Gln residue at the protein terminal, without disturbing its flexibility and

conformation, and modified it with primary amine derivatives of PEGs in the presence of TGase (Figure 2-10).



Gln residue of protein

Figure 2-10: TGase catalysed conjugation

Compared to other methodologies, TGase mediated conjugations are found to be more site-specific, reliable, reproducible and versatile (Fontana et al. 2008). TGase catalysed selective PEGylations of apomyoglobin (apoMb), α-lactalbumin (α-LA), human growth hormone (hGH) human granulocyte colony-stimulating factor (hG-CSF) and human interlukin-2 (hIL-2) with PEG amines are some of the proven examples of this new technique (Fontana et al. 2008; Mero et al. 2009).

2.4.2.5 Miscellaneous conjugation chemistries

DeFrees et al (2006) reported a new site specific process known as GlycoPEGylation, using an enzymatic N-acetylgalactosamine (GalNAc) O-glycolization, followed by PEGylation of the introduced O-glycans using a PEG sialic acid derivative. The emerging new click chemistry strategies (Hein et al. 2008) were are also found to be an important role in PEGylation (Droumaguet and Velonia 2008). Deiters et al (2004) reported a site specific PEGylation of genetically modified superoxide dismutase (SOD) using a PEG-alkyne derivative, resulting in predominant monoconjugation on the azide terminal (Figure 2-11).

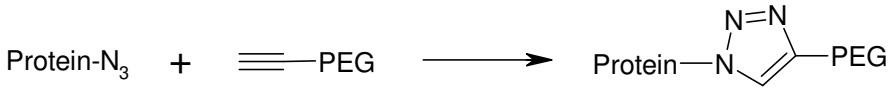


Figure 2-11: Site specific PEGylation using click chemistry (Droumaguet and Velonia 2008)

Even though it has attracted little attention and pharmaceutical relevance, PEGylation of human serum albumin using PEG-phenyl-isothiocyanate (Meng et al. 2008) and PEG-epoxide (Bergstrom and Holmberg 1991) is also cited in literature.

2.4.3 Reversible PEGylation

The improved physicochemical properties of protein PEGylation were overshadowed in many cases by a substantial reduction in the protein activity arising from the permanent linkages formed during PEG conjugation. Consequently, a reversible (or releasable) PEGylation concept is formulated, in which proteins are attached to PEG derivatives through controlled releasable cleavable linkages, which release the protein *in vivo* under physiological conditions at a predetermined kinetic rate (Veronese and Pasut 2009). An example of such reversible PEGylation is the release of PEGylated lysozyme using a 1,6-benzyl elimination mechanism (Lee et al. 2001; Greenwald et al. 2003) (Figure 2-12).

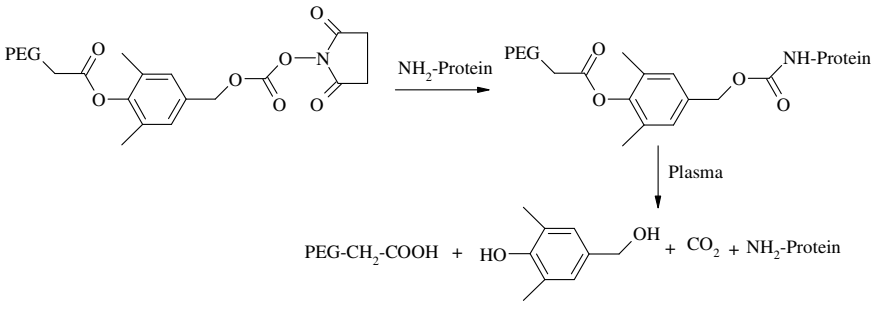


Figure 2-12: Reversible PEGylation using 1,6-benzyl elimination

A number of similar PEG derivatives capable of forming releasable conjugates are also reported, such as bicin, oligo-lactic acid ester, succinic ester, disulfide and β-alanine ester linkers (Filpula and Zhao 2008; Pasut et al. 2008). Even though this technique provided a controlled release of proteins in their fully active forms, the chances of some unwanted residual tags remaining with the protein and the *in vivo* formation of unwanted reaction by-products are some of the areas of great concern with this promising technique.

2.4.4 Structure of PEGs

The development of PEGylation is also characterised by a marked improvement and variation in the nature of the PEGs used for protein conjugation. Early stage PEGylations were performed mainly with linear and low molecular weight PEGs. These PEG derivatives were also characterized by higher diol contents (around 15%) and polydispersity contributed to an increasing heterogeneity of the product profile (Roberts et al. 2002; Pasut and Veronese 2006). The commercial availability of pure and low polydisperse PEG derivatives has resulted in a much improved product profile in recent years.

The introduction of branched PEG derivatives resulted in an improvement in pharmacological properties due to their improved masking and protection of the conjugated protein. The very first branched PEG derivative for protein conjugation was prepared by Matsushima et al. (1980) from the reaction between cyanuric chloride and mPEG and the resulting branched activated species were conjugated with E. coli asparaginase (Figure 2-13). Another branched PEG derivative, namely $mPEG_2$-COOSu prepared by linking mPEG to both α and ε amino groups

of lysine (Monfardini et al. 2002), forms the basic conjugation strategy for manufacturing Pegasys® (Figure 2-6).

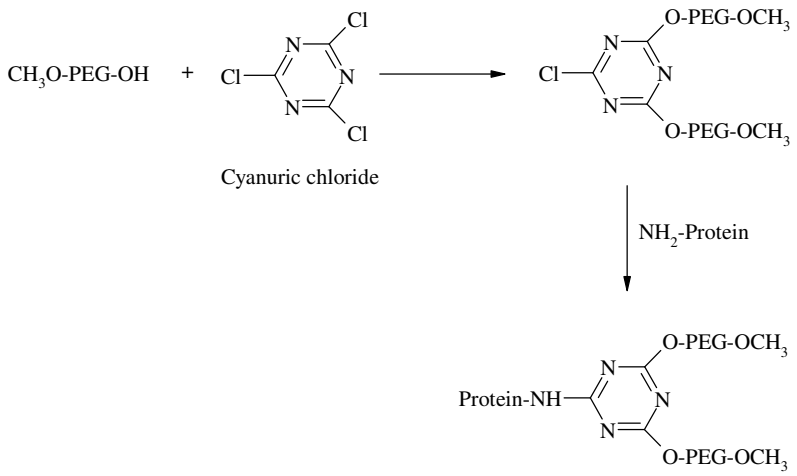


Figure 2-13: PEGylation using cyanuric chloride activated branched PEG

The introduction of PEG dendrimers resulted in further advances in polymer therapeutics, with an increased protection due to the addition of very high molecular weights. In one such instance, Meireles et al (2008) introduced a branched PEG derivative having a dendrimer-like structure with four mPEG branches, with a terminal COOH group available for protein conjugation (Figure 2-14). They successfully conjugated this four-branched PEG dendrimer with a number of therapeutic proteins including IFN-α2b, recombinant streptokinase (r-SK), erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF) and epidermal growth factor (EGF), through NHS activation and found improved pharmacological properties for these products compared with those obtained from two branched-structure of similar molecular mass.

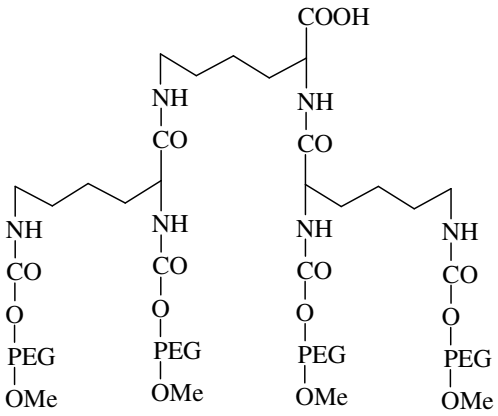


Figure 2-14: Four-branched mPEG-COOH dendrimer derivative

Recent developments in the synthesis of linear and branched multifunctional PEG derivatives, known as Multi-PEGs (Figure 2-15), have contributed a substantial improvement in the pharmacological advantages of PEGylation technology (Schiavon et al. 2004; Pasut et al. 2005; Liu et al. 2007; Zacchigna et al. 2007; Drioli et al. 2008).

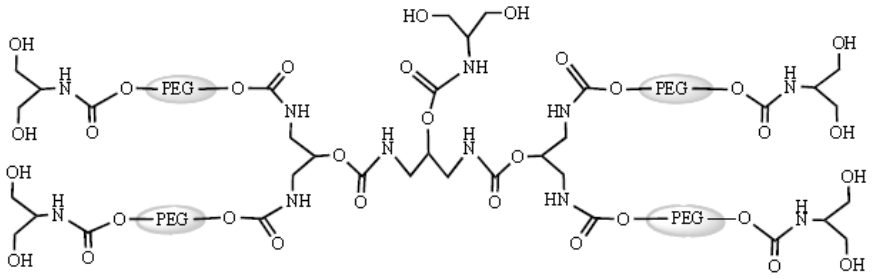


Figure 2-15: A Multi-PEG derivative (TetraPEG$_{6000}$-(OH)$_{10}$) (Drioli et al. 2008)

2.4.5 Process considerations

The overall PEGylation processes so far used for protein conjugation can be broadly classified into two types, namely a solution-phase batch process and an on-column fed-batch process (Fee and Van Alstine 2006).

The simple to use and commonly adopted batch process involves the mixing of all reagents together in a suitable buffer solution, preferably at a temperature between 4–6 °C, followed by the separation and purification of the desired product using a suitable technique based on its physicochemical properties, including sixe exclusion chromatography (SEC), ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and using aqueous two phase systems (Fee 2009).

Normally with the batch process, prolonged contact between reacting species and products results in multiple conjugations and gives rise to a number of PEG isomers. Consequently a heterogeneous product mixture results, constituting unreacted starting materials, hydrolysed activating agents and a wide range of PEGylated product with varying degrees of conjugation. Hence extensive multistep purifications and downstream processing are required to isolate the desired product (Fee and Van Alstine 2006), making this process commercially non-viable and less attractive in many cases. The high cost of the therapeutic proteins, along with the cost of separating the desired PEGylated protein from these resulting mixtures, makes the products extremely expensive. A typical SEC elution profile for various PEGylated α-lactalbumin products obtained from a batch process is illustrated in Figure 2-16.

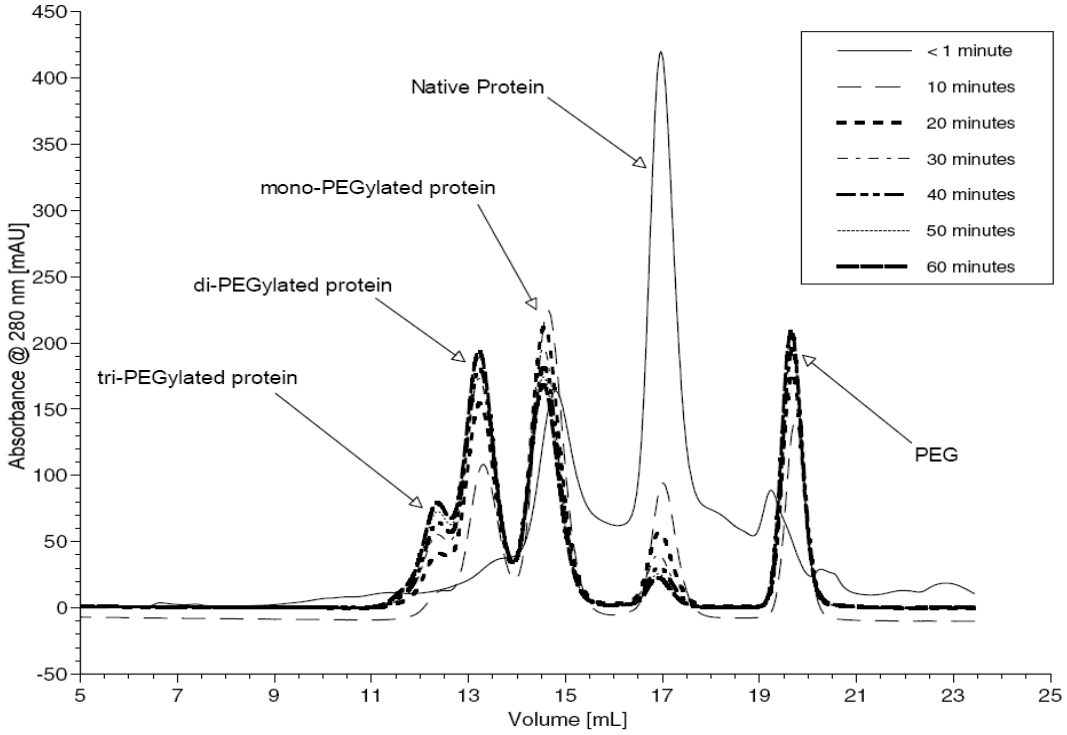


Figure 2-16: SEC elution profile of various PEGylated α-lactalbumin products obtained from batch process (Fee 2003)

Taking into considering the improvement of the product profile, a number of on-column PEGylation techniques were attempted, mainly to improve the specificity of the conjugation. The very first variation in the PEG conjugation process was introduced by Felix et al (Lu and Felix 1994; Felix 1997). They developed a site-specific solid-phase peptide PEGylation, in which a peptide sequence was tethered onto a Rink amide MBHA-resin and was conjugated with a PEG derivative through a side chain lysine or aspartic acid. Finally the mono-PEGylated peptide was cleaved off from the resin using trifluoroacetic acid (TFA). In a similar pattern, folate was derivatised with a PEG derivative attached to HMP resin through a peptide linker and peptide-PEG-folate conjugate was cleaved off from the resin using TFA (Niculescu-Duvaz et al. 2008). Because of the utilization of very harsh chemicals like TFA for the release of PEGylated products, direct application of this methodology is not viable with highly sensitive species like proteins.

Utilizing the ion exchange interactions between protein and ion exchange resins, Monkarsh et al (1997) prepared and separated various positional isomers of PEGylated interferon-α-2a (PEG-INF) using an on-column process. INF was first adsorbed to a strong anion exchange resin and the activated PEG derivative was then circulated through the column. After eluting the unreacted PEGs and by-products, PEG-INF was collected by increasing the salt concentration. Unfortunately, this method resulted in multi-PEGylated products without any improvement in the product profile.

Similarly, Lee et al (2007) PEGylated recombinant interferon-α-2a (rhIFN-α-2a) by adsorbing on a CM-Sepharose cation exchange resin through ion exchange interactions. After washing out the unbound rhIFN-α-2a, mPEG-aldehyde and sodium cyanoborohydride solutions were

passed through the column. Similarly Suo et al (2009) PEGylated bovine haemoglobin loaded onto CM-Sepharose using mPEG-succinimidyl carbonate and their studies showed that the selectivity of this method was increased with an increase in the molecular weight of the added PEG. A schematic illustration of the concept of their on-column "solid-phase PEGylation" is illustrated in Figure 2-17. Even though these methods resulted in preparing mono-PEGylated products up to 75% yield, based on the loaded native protein, consistency in the reaction efficiency is highly unlikely due to random orientations of adsorbed protein on the ion exchange matrix.

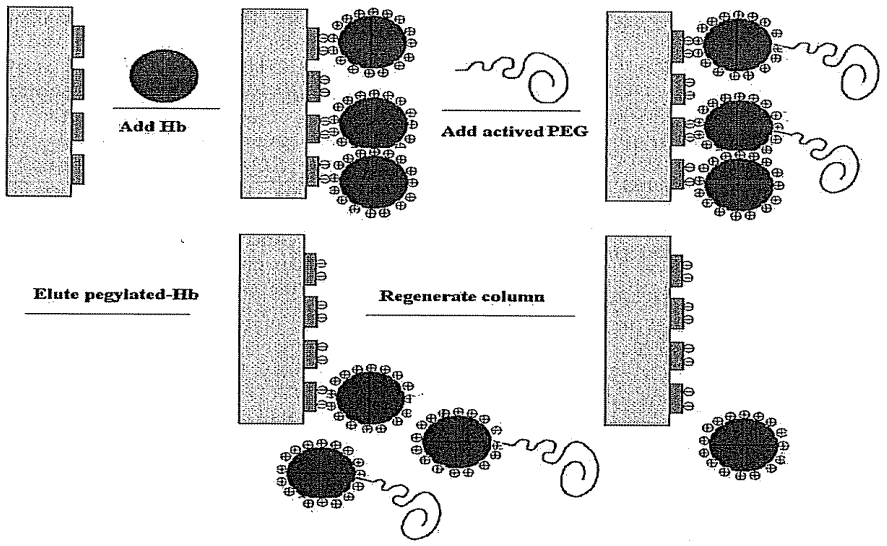


Figure 2-17: Schematic illustration of on-column solid-phase PEGylation based on ion exchange interactions (Suo et al. 2009)

A unique on-column PEGylation methodology, known as size exclusion reaction chromatography (SERC), was introduced by Fee (2003), incorporating the principle of SEC in separating various molecular sized species based on their different linear velocities through a column packed with porous beads (Figure 2-18). In this method, both activated PEG and protein were injected into the column, which subsequently forms an *in-*

situ moving reaction zone within the column. As the reaction proceeds, owing to the increased linear velocity associated with the larger molecule, the PEGylated protein, having a larger size than either of the reagents, moves ahead of the reaction zone. Consequently a simultaneous reaction as well as the separation of the various PEGylated products can be achieved in a single operation.

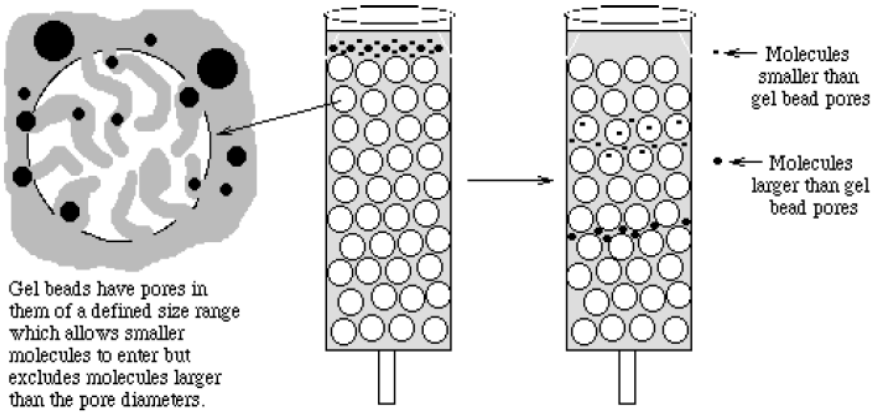


Figure 2.18: Principle of SEC
(http://www.sci.sdsu.edu/TFrey/Bio750/Chromatography.htm)

Unfortunately, SEC is characterized by its own inherent inability to identify and separate the various positional isomers with the same number of PEG molecules attached through the different conjugation sites of a given protein. This drawback also extended to SERC, even with its other advantages.

2.4.6 Requirement for an improved technique

Even though nearly four decades of development of PEGylation technology have proved its pharmacological advantages and acceptability, the technology still lags in providing a commercially attractive and versatile process to yield the desired products with high

specificity, quantity and quality. Being a multi-million dollar business annually, and with the growing influence of emerging biotechnology and the interest of multinational pharmaceutical companies, there is a great scientific and commercial interest in improving on the various drawbacks of the present methodologies and in the need for innovative process variations.

2.5 Concept of novel solid-phase protein PEGylation

In order to overcome these drawbacks, a novel and innovative solid-phase protein PEGylation design is proposed, with its characteristic unidirectional and single pass process to minimise the variation between batches and to increase the specificity and yield of the process. The new design, solid-phase PEGylation resin, involves the use of a tethered PEG molecule on a modified solid matrix. A schematic representation of the proposed solid-phase PEGylation matrix is illustrated in Figure 2-19. Because of the high mobility of the end terminal of the tethered PEG molecules and due to the presence of inter-repulsive forces exerted between the hydrated PEG chains, there is a strong possibility of single PEG molecule conjugation with the approaching protein. Consequently a predominant mono-PEGylated product can be expected with a reduction in product heterogeneity, allowing the production of PEGylated species with high yields and purity, which significantly reduces separation and purification requirements.

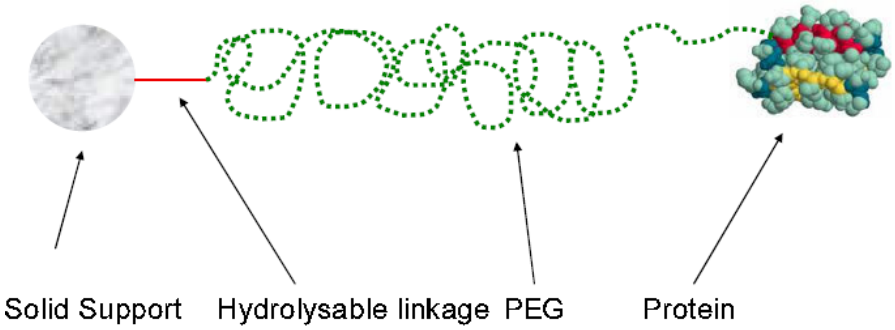


Figure 2-19: Schematic representation of the concept of solid-phase PEGylation system

The solid-phase PEGylation technique involves the grafting of a functionally modified PEG onto a functionalized solid matrix through a pH-sensitive labile linkage. The free end terminal of the PEG is modified with a functional group capable of selective conjugation with a particular functional group available on the protein surface. After protein conjugation, the PEGylated protein will be released from the solid support by cleaving the labile linkage, by a mild change in pH or the use of a suitable reagent, without disturbing the protein-PEG linkage or denaturing the protein. This novel solid-phase PEGylation matrix is anticipated to have the following characteristics:

1. It can be used for both batch and on-column processing.
2. It reduces heterogeneity and improved product profile.
3. It minimises the variation between batches and increase the specificity and yield of the process.
4. The amount of protein required for conjugation can be calculated based on the activated PEG on the solid matrix, which results in a reduction in the wastage of protein.

5. It allows recycling of unreacted and excess protein for further conjugation.
6. The end terminal functional group of the tethered PEG can be easily modified to adapt this technique for a wide variety of site specific PEGylation strategies.
7. It is ready to use and can be stored under inert atmosphere.

2.6 Components of solid-phase protein PEGylation matrix

The proposed solid-phase protein PEGylation matrix can be divided into three main components, the functionalized matrix, a cleavable linker and a heterobifunctionalized PEG derivative. Here follows a brief discussion about the choice of these various components, along with the model protein Cytochrome c, more relevant discussions particularly related to the project can be find in the specifically concerned chapters.

2.6.1 Cytochrome c

Cytochrome c from bovine heart is used as the model protein for conjugation studies. Because of the presence of a homologous structure (Margoliash 1963) and its characteristic electron-transferring capabilities (Mirkin et al. 2008), Cytochrome c is one of the most extensively used model globular proteins. Cytochrome c is characterised by a single polypeptide chain consisting of 104 amino acids covalently attached to a heme group and having a molecular weight of 12,130 Da and a hydrodynamic radius of 3.4 nm. As Cytochrome c is a lysine rich protein with 19 lysine residues distributed on the protein surface (Prakash and Mazumdar 2009) (Figure 2-20), amine reactive conjugation strategy can be easily applicable for any modifications.

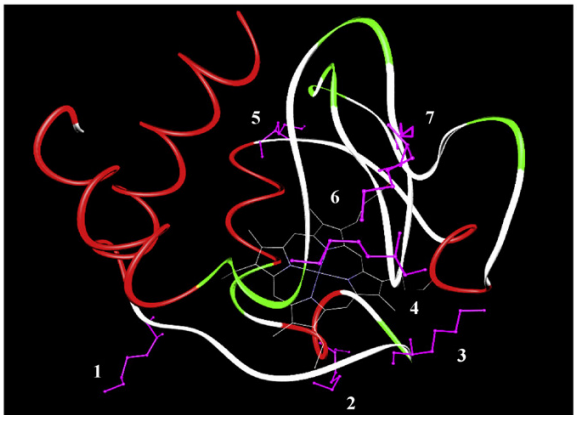


Figure 2-20: Structure of Cytochrome c showing reactive lysine residues (Prakash and Mazumdar 2009)

2.6.2 Functionalized solid matrix

The basic requirement of this matrix is to provide a structural support for the functionalized PEG derivative during protein conjugation, without denaturing the protein or interfering with conjugation reactions. Examples of various solid-phase support matrices with a special emphasis on the immobilization of biomolecules include cross linked natural organic polymers such as agarose (O'Shannessy 1989; Manta et al. 2003), dextran (Veronese and Morpurgo 1999; Mehvar 2000), cellulose (Bora et al. 2005), chitin and chitosan (Chan et al. 2007); synthetic polymers like polystyrene (Zalipsky et al. 1994; Van Delden et al. 1996; Steven A. Kates et al. 1998), polypyrrol (Dong et al. 2009) and polymethacrylate (Chan et al. 2007); inorganic polymers such as silica and glass (Choi et al. 2005), and some functionalized nanoparticles (Jiang et al. 2004; Yang et al. 2007). These matrices are used in various forms such as beads, solid surface and membrane, depending on the purpose.

In this thesis, experiments are planned mainly using two different types of solid matrices, namely hydrophilic and hydrophobic matrices, to

understand their influence on the nature of the novel PEGylated system. Sephadex™ (GE Healthcare Ltd, Uppsala) is selected for the former class and polystyrene is selected for the latter one.

2.6.2.1 Sephadex

Sephadex is a crosslinked dextran that is widely used as a chromatographic medium for the separation and purification of a wide variety of biomolecules. A variety of Sephadex derivatives is available in the market, based on the difference in their cross linking and the degree of swelling. Sephadex derivatised with cation exchangers, such as carboxymethyl Sephadex (CM Sephadex), sulfopropyl Sephadex (SP Sephadex) and anion exchangers such as diethylaminoethyl Sephadex (DEAE Sephadex) and diethyl-(2-hydroxypropyl) aminoethyl Sephadex (QAE Sephadex) are also available for chromatographic purposes.

Sephadex is prepared by cross linking low molecular weight dextran with epichlorohydrin in the presence of alkali (Flodin 1962; Flodin 1998). The resulting gel is then neutralized, ground, dehydrated, dried and sieved to isolate the desired particle sized fractions. Dextran, the basic structural component of Sephadex, is a complex branched glucan (α-D-glucopyranose) with mainly α 1-6 glycosidic linkage between glucose molecules, and about 5% α 1-4, α 1-2 and α 1-3 linkages. The reactivity of various OH groups in the dextran molecule varies with their position, and studies showed that the OH group on C2 position is more reactive than other OH groups (Ramirez et al. 1994; Lee et al. 1999). This is mainly due to the presence of neighbouring oxygen atoms in the ring and on the C1 position, which enable the interaction of their lone pair of electrons through space, resulting in an increased electron density at the C2 position and thereby making the OH group more nucleophilic. This

type of cross linking with epichlorohydrin will occur mainly at the C2 positions between the dextran moieties and the basic structure of Sephadex can be presented as shown in Figure 2-21. The remaining OH group at C2 positions, left behind after cross linking, are the major targets for further modifications.

The cross linking of dextran base units results in the formation of well defined three dimensional networks of interstices or pores with a large number of hydroxyl groups, making these gels polar, and consequently these gels swell significantly in water and aqueous solutions (Flodin 1962; Vavruch 1968).

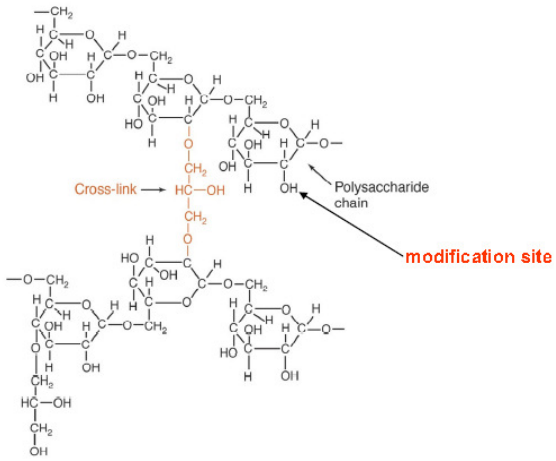


Figure 2-21: Basic structure of Sephadex (Flodin 1962)

2.6.2.2 Polystyrene

The introduction of cross-linked polystyrene for the solid-phase peptide synthesis by Merrifield (1963) attracted an overwhelming interest in solid-phase chemistry. In contrast to Sephadex, the absence of any hydrophilic and polar functional group on the polystyrene polymer chain (Figure 2-22) makes it hydrophobic and results in good swelling

properties with organic solvents such as dichloromethane and dimethyl formamide (Park and Lee 2000).

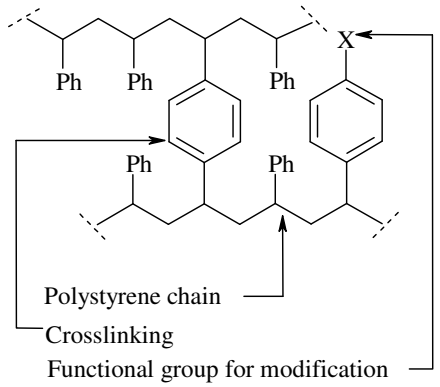


Figure 2-22: Basic structure of polystyrene (Campbell 2000)

2.6.3 Cleavable linker

A cleavable linker between the matrix and the PEG derivative is anticipated to enable the release of PEGylated product from the matrix after protein conjugation. Even though a large variety of cleavable linkers are available for conventional solid-phase and combinatorial synthesis (Guillier et al. 2000), the choice of a suitable linker for solid-phase PEGylation was limited by the presence of protein in the reaction system. Very mild cleavage conditions were the primary requirement considering the very high denaturing tendency of the conjugated protein and consequently labile ester linkages were selected (Section 3.2.1.1).

As an alternate to labile ester linkages, the use of selective and site specific proteins tags (Terpe 2003) also attracted attention while drafting the methodology. Due to the very specific selectivity of these recognition sites by cleaving enzymes, these tags were found to be very important in protein expression and purification as well as protein synthesis (Bang and

Kent 2005; Gaberc-Porekar and Menart 2005). Some commercially available cleavage enzymes and their recognition sites are summarized in Table 2-3. The insertion of such a tag between the resin and one terminal of the PEG derivative is a good option to improve the yield and selectivity of the PEG release, as well as to reduce the chances of denaturing the protein

Table 2-3: Examples for recognition site and enzymes (Gaberc-Porekar and Menart 2005)

Recognition site	Cleavage enzyme
Ile-Glu/Asp-Gly-Arg▼	Factor Xa
Leu-Val-Pro-Arg▼Gly-Ser	Thrombin
Leu-Glu-Val-Leu-Phe-Gln▼Gly-Pro	PreScission
Asp-Asp-Asp-Asp-Lys▼	Enterokinase
Glu-Asn-Leu-Tyr-Phe-Gln▼Gly	TEV protease
Leu-Glu-Val-Leu-Phe-Gln▼Gly-Pro	HRV 3C protease

2.6.4 PEG

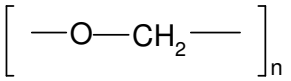
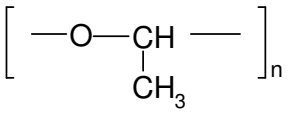
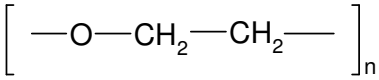
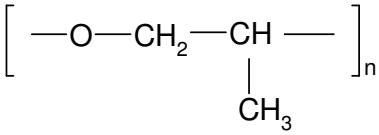
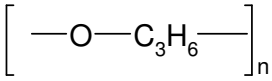
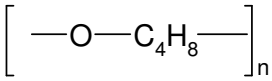
Considering the influence of PEG polymer on the overall preparation and performance of the proposed protein delivery system, it is worthwhile to understand the characteristic properties of this polymer, especially the structural features.

2.6.4.1 Properties of PEG molecule

PEGs are generally prepared by the ring opening polymerization of ethylene oxide (Hill et al. 1958; Bailey and Koleske 1976). Compared to its structural analogues, PEG has very good water solubility under

ordinary conditions (Kjellander and Florin 1981; Israelachvili 1997) (Table 2-4).

Table 2-4: Solubility of PEG and similar polymers in water

Polymer	CAS No	Structure	Solubility in water
Poly (methylene oxide)	9002-81-7		Insoluble
Poly (acetaldehyde)	9002-91-9		Insoluble
Poly (ethylene oxide)	25322-68-3		Soluble
Poly (propylene oxide)	25322-69-4		Insoluble
Poly (trimethylene oxide)	31714-45-1		Insoluble
Poly (tetrahydrofuran)	25190-06-1		Insoluble

The high solubility of PEG in water at ambient temperature can be mainly attributed to the conformational behaviour of the ethylene oxide fragment of the polymer backbone (Section 2.6.4.2.1). PEG water solubility is characterized by a distinctive inverse solubility-temperature relationship (Bailey and Koleske 1976); as the temperature increases the polymer starts precipitating out from the solution. This is known as the clouding effect and the temperature at which the PEG starts precipitating

out (cloud point or the lower critical solution temperature (LCST)) depends on the molecular weight of PEG, its concentration, the presence of any salts and the pH of the solution (Han and Jhun 1984; Armstrong et al. 2001; Nozary et al. 2003). Jeon and Chang (1996) correlated this behaviour to the intrinsic viscosity of the PEG-water solution. Their studies showed that the intrinsic viscosity of the PEG-water solution decreases with an increase in temperature, and suggest that this results in a decrease in the PEG-water interaction, which contracts the PEG chain and thereby induces hydrophobic interactions.

PEG is also soluble in a variety of organic solvents like chlorinated hydrocarbons, but insoluble in hexane, diethyl ether and ethylene glycol (molecules which resemble PEG). Even though PEG is soluble in both organic and aqueous solvents, the partitioning property of PEG will vary depending on the nature of the solvent (Harris 1992). In a water-benzene system, PEG will partition in favour of water, whereas in a water–dichloromethane system, it favours dichloromethane. Also by taking advantage of the reduced solubility of PEG in aqueous solutions in the presence of salts (Armstrong et al. 2001), this favourable partition coefficient towards dichloromethane can be used as an effective methodology in avoiding the formation of emulsions usually associated with extracting PEGs from their aqueous solutions using organic solvents. Consequently, PEG derivatives extracted from a saturated NaCl solution using dichloromethane were adapted for the workup during their synthesis (Section 3.5).

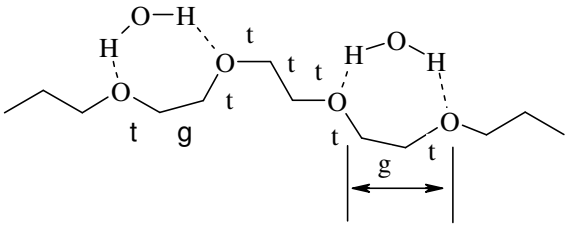
2.6.4.2 PEG Conformations

The conformational behaviour of PEG molecule varies depending on a number of factors. If the molecule is in solution, the nature of the

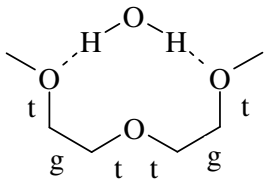
medium was found to have a great influence on the conformational property of PEG molecules. If the polymer is attached to a matrix surface, the conformational behaviour is greatly influenced by surface PEG concentrations.

2.6.4.2.1 PEG conformation in solutions

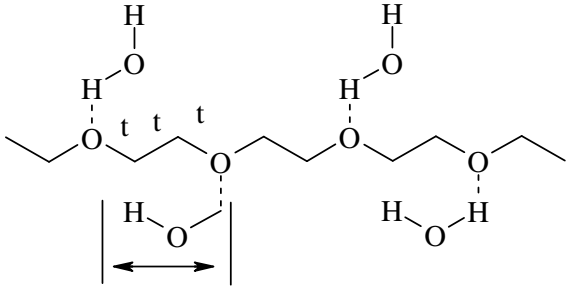
In aqueous solutions, PEG can exist in either trans-gauche-trans (t-g-t) or all trans (t-t-t) conformations, as shown in Figure 2-23.



a.1) tgt-ttt conformation



a.2) tgt-tgt conformation



b) ttt conformation

Figure 2-23: PEG conformations (Rixman et al. 2003)

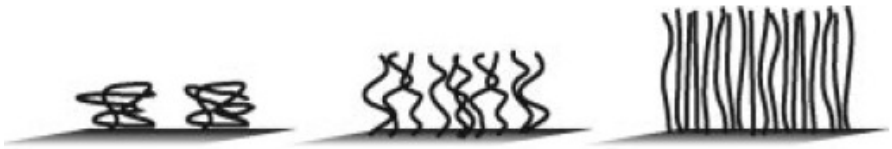
In t-g-t conformations, a single water molecule can form hydrogen bonding between the two adjacent oxygen atoms in PEG, resulting in the formation of bridges that stabilize the dynamic helical configuration. This is further favoured by a structural matching between the ethoxide moiety

and water, with a comparable distance between the oxygen atoms in -O-CH_2-CH_2-O- segments in the gauche conformation and the hydrogen atoms within the nearest water molecule (Begum and Matsuura 1997). In the t-t-t conformation, the increased distance between oxygen atoms (Figure 2-23b) prevents the bridge formation and allows only one hydrogen bond between the water molecule and the polymer backbone. Consequently, there is an increase in the mobility of the molecule and therefore the t-t-t configuration is favoured by an entropic advantage (Oesterhelt et al. 1999).

Because the chances of such hydrogen bonding are almost negligible with aprotic solvents, PEG will exist in an all trans conformation in such solvents, leading to the formation of random coils, but the presence of a small amount of water can favour helical formations. Thus a polymer exists predominantly in its random coil form in organic aprotic solvents and in helix form in aqueous solutions. Also, at high molecular weights, PEG exists as a helix in its solid state and when it is melted, the destruction of the helical structure takes place, leading to the formation of random coils. Studies on the heat of fusion, ΔH_f, of PEG with a molecular weight of 6000 Da in water (74.5 J/g) and organic solvents such as chloroform and dichloromethane (239 J/g) (Maron and Filisko 1972), showed a difference of 164.5 J/g of polymer, confirming the difference in conformation of PEG in these solvents and suggesting the existence of a helical conformation in water and a random coil conformation in organic solvents. This also suggests that this quantity of heat is required to disrupt the dynamic helical configuration, and the high ΔH_f value for PEG in non-aqueous solvents may be mainly due to the increased disorder introduced by the conversion of helix to random coils.

2.6.4.2.2 Conformational behaviour of surface-grafted PEG

The conformational behaviour of surface-grafted PEGs, derived from the theories initially proposed by Alexander (1977a; 1977b) and de Gennes (1980; 1987) and by many others in recent years (Kuhl et al. 1994; Vermette and Meagher 2003; Norde 2007; Wattendorf and Merkle 2008; Steinmetz and Manchester 2009), suggests the existence of a variety of conformations, ranging from mushroom to brush forms with overlapping regimes.



a) Mushroom b) Brush-mushroom intermediate c) Brush

Figure 2-24: Schematic representation of various conformations of surface grafted PEGs (Wattendorf and Merkle 2008)

de Gennes, in his theory (1980; 1987), correlated grafting density to preferred conformations and suggested that a mushroom conformation exists at a low graft density, while a high grafting density will favour a brush conformation. With lower grafting densities, the distance between grafting terminals of the PEG chains (D) will be greater than the Flory radius (R_F) of the polymer ($D > R_F$), allowing mushroom conformations. As the grafting density increases, the distance between the grafting terminals will decrease, causing the polymer chains to stretch away from the matrix due to interchain interactions. In this case, $D < R_F$, and the polymer will be predominantly in its brush conformation.

2.7 PEG grafted solid matrices

PEG grafted matrices are used in a variety of applications, such as solid-phase peptide synthesis (Felix 1997), enzyme immobilization (Manta et al. 2003), anti-fouling surfaces (Malmsten and Muller 1999; Xu et al. 2008), biosensors (Sharma et al. 2004; De Vos et al. 2009), molecular brushes (Gao and Matyjaszewski 2007; Halperin et al. 2007) and ligand spacers (Ham et al. 2009). The improved properties of these modified matrices are mainly attributed to the unique nature of PEG molecules (Section 2.3). Various methods of preparing these grafted matrices are described in following sections.

2.7.1 Physisorption

A variety of PEG-matrices can be prepared by physical adsorption of PEG onto surfaces based on electrostatic interactions. A large number of such materials have proved their importance in biomedical and chromatographic applications due to their simple method of preparation, but were characterized by their non-specificity and lack of permanent intact on the surface (Lee et al. 1995). Examples include the adsorption of PEG onto poly(ethylene imine) functionalized silica and mica surfaces (Claesson et al. 1996) and through poly(L-lysine) derivatization on a negatively charged metal oxide substrate (Pasche et al. 2005).

2.7.2 Block polymerization ("*grafting from*")

PEG grafted solid matrices using block polymerization are prepared by using PEG as the polymerization macroinitiator, functionalized with an initiation group capable of starting the polymerization of a second monomer (Borner and Matyjaszewski 2002). Depending on the structure

of the monomer units, the polymerized products displayed characteristic sequences and polymer chain networks, resulting in the formation of mono-block, di-block or multi-block copolymers (Xie and Xie 1999). Solid matrices with high grafting density molecular brushes can be conveniently prepared by this method (Borner and Matyjaszewski 2002).

A number of examples for preparing PEG grafted matrices using block copolymerization are cited in literature (Xu et al. 2006; Durmaz et al. 2008; Varshney et al. 2008). Ring opening copolymerization of ethylene oxide (EO) with ethoxyethyl glycidyl ether (EEGE) in the presence of mPEG yielded a linear macroinitiator that initiated the atom transfer radical polymerization (ATRP) of styrene. The resulting comb-like polymer (mPEG-b-[poly(EO-co-Gly)-g-PS]) is a classic example of a PEG-block copolymer prepared by using the *graft from* polymerization strategy (Sun et al. 2009).

2.7.3 Graft polymerization (“*grafting to*”)

PEG grafted matrices through graft polymerizations are mainly prepared by the utilization of free radicals, peroxides or various high energy irradiations (Lee et al. 1995). PEG methacrylate or azide derivatives are commonly used for this type of grafting, due to their capability for direct insertion into C=C or C-H bonds (Thom et al. 1998). Consequently this methodology offers PEG grafting onto a variety materials, independent of the surface composition or chemistry. Examples include the grafting of PEG onto gold (Larsson et al. 2007), stainless steel (Zhang et al. 2001), polysulfone (Thom et al. 1998), polystyrene (Lazos et al. 2005) and poly(vinyl chloride) surfaces (Lee et al. 1995).

2.7.4 Covalent grafting

The covalent grafting of a PEG onto a matrix is a type of "*grafting to*" technique that provides the most effective method of creating a permanent linkage between the matrix and the polymer. Well defined tailor made PEG derivatives capable of forming covalent bonding with the available functional groups on the matrix surface and the use of ambient reaction conditions, make this technique more attractive than the other methods. In the case of matrices without any functional groups, a pre-modification of the surface with a suitable functional moiety is required before PEG grafting. A detailed insight into this method is given in Section 4.2.2.

2.8. Protein interactions with PEG grafted matrices

Proteins can be considered as complex polymers made of various amino acids (Branden and Tooze 1991). Consequently, their interaction with another polymer grafted matrix surface will result in a variety of simultaneous interfacial interactions consisting of many attractive and repulsive forces, depending on PEG grafting density, chain length and the nature of approaching proteins (Jeon and Andrade 1991; Jeon et al. 1991; Leckband and Sivasankar 1999; Vermette and Meagher 2003). The overall outcome of these forces in general leads to two types of protein adsorption on a PEG grafted solid matrix, namely primary and secondary adsorptions (Szleifer 1997; Halperin 1999). A primary adsorption is characterized by a primary potential energy minima close to the matrix surface, whereas a secondary adsorption occurs at a secondary potential energy minima, usually farther away from the surface (Vermette and Meagher 2003) (Figure 2-25).

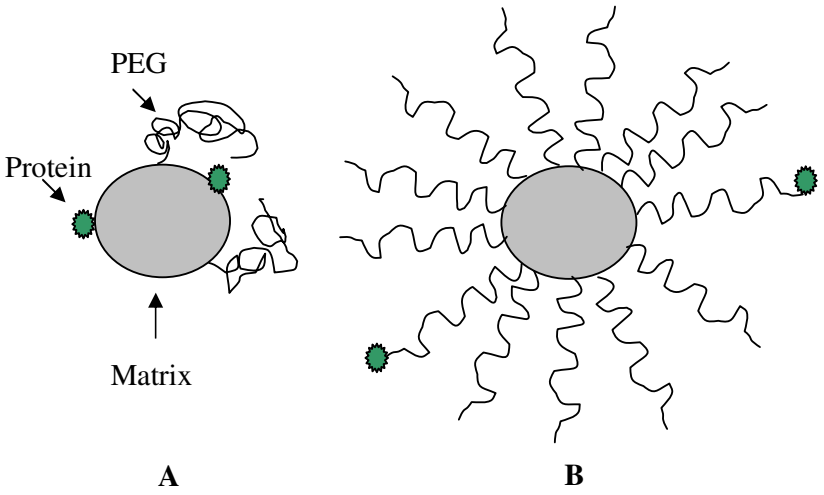


Figure 2-25: Schematic illustration A) Primary adsorption and B) Secondary adsorption of proteins

A brief survey of various possible interactions that can influence the performance of the novel solid-phase PEGylation system are summarized below.

2.8.1 Attractive interactions

In aqueous solutions PEG chains can undergo partial oxidations, resulting in the development of small negative charges along the polymer chain, and proteins can be positively charged at physiological pH. This can contribute towards the formation of an attractive electrical double layer interaction between the PEG molecules and the approaching protein (Vermette and Meagher 2003). If the PEG grafting density is low and the underlying matrix surface is characterised by negatively charged functional groups, these attractive interactions can lead to the formation of mainly primary adsorptions on the matrix surface. Similarly some other short range attractive forces, including hydrogen bonding and van der Waals interactions (Rixman et al. 2003), may also be exerted between the matrix surface and the approaching protein during conjugation.

2.8.2 Repulsive interactions

According to de Gennes (1987), surface grafted PEG molecules in their brush conformation can be treated as a polymer solution whose concentration will increase with an increase in grafting density. An increase in the concentration of this solution will result in an apparent increase in osmotic pressure between the chains, due to an increase in polymer chain segment concentration with a decrease in the elastic restoring force arising from the configurational entropy (Jeon et al. 1991; Rixman et al. 2003). When a protein approaches such a highly concentrated solution, there will be an increased repulsive force (F) contributed by both osmotic pressure and elastic restoring forces as in Equation 2-1 (de Gennes 1987)

$$F \approx \frac{kT}{D^3}\left[\left(\frac{2t}{h}\right)^{9/4} - \left(\frac{h}{2t}\right)^{3/4}\right] \qquad (2\text{-}1)$$

where k is the Boltzmann constant, T is the absolute temperature, D is the distance between the grafted polymer chains, t is the thickness of the PEG layer and h is the average distance between protein and polymer grafted matrix. The first term in Equation 2-1 outside the bracket represents the osmotic term, while the second one is the elastic term. This indicates a complete domination of the osmotic factor, resulting from compressions occurring due to approaching proteins for conjugation. Simultaneously, these compressions and the resultant outward osmotic pressure tends to remove water molecules out from the PEG hydrated shells. This results in thermodynamically unfavourable conditions causing increased steric repulsions, and finally contributes towards an inertness to PEG molecules (Ostuni et al. 2001).

2.8.3 Impact of various surface force interactions on expected results

Considering all these cumulative surface force interactions, the magnitude and range of repulsive interactions are much higher than those of attractive interactions and will increase with an increase in PEG grafting densities. Consequently with a higher grafting density, where the grafted PEG molecules will predominantly be in their brush conformations, a localised higher concentration of protein can be expected near the terminal end of the PEG molecules, due to secondary potential energy minima. Therefore, this will result in the conjugation of activated PEG terminals with proteins accumulated on the secondary potential energy minima zone nearer to the PEG terminals (Figure 2-26). Taking advantage of the high mobility of the PEG end terminal as well the repulsive interactions between the hydrated PEG chains, this keeps the polymer chains apart from each other; a predominant mono-PEGylation is anticipated as an outcome of the resulting conjugation.

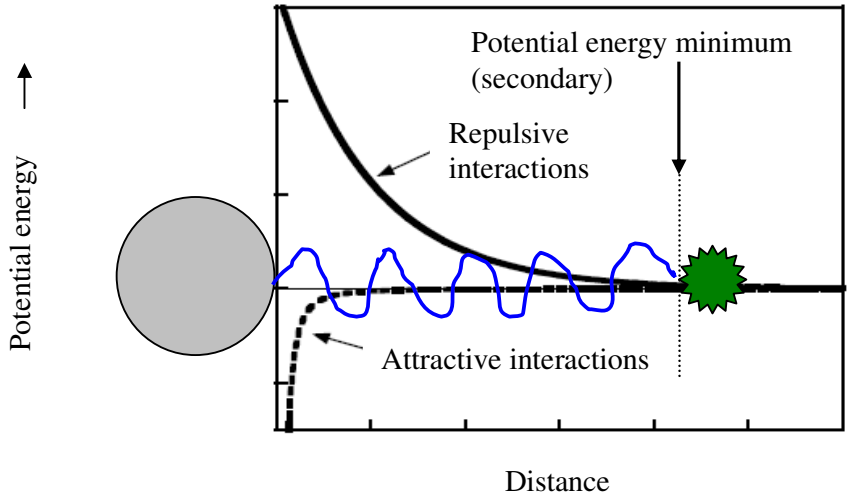


Figure 2-26: Influence of surface interactive forces on solid-phase protein PEGylation

2.9 Conclusions

Improved pharmacological advantages and the commercial acceptance of various PEGylated products catalyzed a substantial improvement in their chemistry and process. Even though the introduction of various site specific conjugation techniques and process variations can improve the product profile and yield of PEGylation, there are very good possibilities for further improving this technology to overcome the existing challenges and to meet commercially acceptable norms.

After going through a detailed insight into various conjugation strategies and process considerations and evaluating the present limitations and drawbacks, the requirement for an improved technique is ascertained in this chapter. The proposed methodology, solid-phase protein PEGylation, utilizes the concept of surface force interactions between the grafted PEG molecules and the approaching protein, and is anticipated to provide an improved product profile compared with other methods by reducing the product heterogeneity and variation between the batches.

3 Synthesis of heterobifunctional PEG derivatives

3.1 Introduction

This chapter describes the various methodologies used for synthesizing suitable PEG derivatives for surface grafting, protein conjugation and hydrolysis kinetic studies. Solution-phase protein PEGylation using prepared PEG derivatives is also described to compare the selectivity of the conjugation and nature of product profile with its solid-phase analogue.

The development and optimization of a suitable synthetic scheme for a heterobifunctional PEG derivative which suits the requirements of the current project objectives was not straightforward due to the lack of literature and reported methodologies. The synthetic schemes for PEG modifications were finalized based on similar functional group transformations and from the results of various experiments with a number of reaction schemes, chosen for their compatibility with the different functional moieties present on either side of the PEG molecule during their preparation and modifications.

3.2 Synthesis strategy development

3.2.1 PEG derivative for solid-phase protein conjugation

One of the main objectives of the project was to develop a suitable PEG derivative with a functional group capable of forming a cleavable linkage with a functionalized solid matrix at one terminus and the other end of the PEG chain incorporating a functional moiety capable of forming a

stable linkage with a suitable functional group on a protein under mild conditions. Once the PEG derivative was grafted onto the matrix, the other terminal would conjugate to the protein and the PEG-protein conjugate would finally cleave off the matrix to yield a PEGylated protein with a desired degree of PEGylation.

3.2.1.1 Choice of a suitable cleavable linkage

Cleavable linkers are commonly used with solid-phase synthetic strategies and their choice depends on the reaction parameters and nature of the product required. A brief summary of the various labile linkages used in organic preparations with a special emphasis on solid-phase synthesis is summarised in Table 3-1.

In the majority of reported cases, very harsh reaction conditions and chemicals were utilized for cleaving the linkage and the resulting hydrolysed product ends up with a residual terminal functional group from the cleaved linkage. The ultimate goal of this project was to produce PEGylated proteins and this, along with the presence of protein in the reaction media during the cleaving process, necessitates the use of very mild cleavage conditions and reagents to prevent denaturing of the protein. Also, any resulting PEGylated product with a residual reactive terminal functional group can induce toxicity *in vivo* and hence will not be approved as a drug component by the drug regulation authorities. Considering all these limitations and requirements, an ester linkage was selected, because the cleavage of an ester will result in a less reactive hydroxy terminated PEG derivative and it was anticipated that this could be performed under very mild conditions with low toxicity chemicals.

Table 3-1: Labile linkages

Linkage	Nature	References
Acetal R-CH-$(OR')_2$	Acid labile	(Cordes and Bull 1974; Furman et al. 1999; Rozema and Wakefield 2005; Trubetskoy et al. 2005)
Carbamate (urethane) R_2-N-CO-O-	Labile, nature varies with substituents	(Letsinger et al. 1964; Dressman et al. 1996)
Diazo -N=N-	Cleavage by dithionite	(Jaffe et al. 1980; Hermanson 1996)
Disulfide -S-S-	Cleavage via reduction	(Hermanson 1996; Salo et al. 1998; Lack et al. 2002)
Enamine R_2C=CR-NR_2	Acid labile	(Hird et al. 1997; Dorwald 2003)
Enol ether R_2C=CR-O-R	Acid labile, cleavable by oxidising agents (eg. dimethyl dioxirane)	(Dorwald 2003; Roberts and Hartley 2004)
Esters -CO-O-	Acid and base labile	(Greenwald et al. 1996; Dorwald 2003)
Imine -N=C-	Acid labile	(Baker et al. 1996; Kim et al. 2005)
Ketals R_2-C-$(OR')_2$	Acid labile	(Cordes and Bull 1974; Olsen et al. 1985)
Orthoester $RC(OR')_3$	Acid labile	(Cordes and Bull 1974)
Silyl ether -O-SiR_2-	Acid labile	(Boehm and Showalter 1996; Ishii et al. 2000; Wang et al. 2001)

Table 3-1 continued

β-thiopropionate -O-CO-CH_2- CH_2-S-	Acid labile	(Oishi et al. 2005)

3.2.1.2 Succinyl ester as a cleavable linker

Succinyl ester is a useful cleavable linker for attaching drugs to many polymeric delivery systems due to the ability to hydrolyse it under very mild conditions (Zalipsky et al. 1983; Douglas et al. 1991; Plourde et al. 1995; Braunova et al. 2004). Introduction of a succinyl ester between the functionalised solid matrix and one terminal of PEG was thus considered to be a good option to meet and overcome many of the project requirements and constraints, respectively.

Detailed synthetic methods and results are attached in Appendix A. In brief, one terminal of the PEG was first functionalized with a carboxy derivative, followed by protecting it with a suitable functional group to make it available for further activation and conjugation with protein. The unmodified hydroxyl terminal was then esterified with succinic anhydride and grafted onto Sephadex under anhydrous conditions. The carboxy terminal of the PEG was regenerated by deprotection and then conjugated with protein after NHS activation. Finally, the objective was to cleave off the PEGylated protein by hydrolysing the succinyl ester linkage under very mild reaction conditions. The hydrolysed fractions were analysed using SEC chromatography but the results were negative - no PEGylated protein was detected in the hydrolysed fractions. Experiments were repeated with various protection-deprotection methods and various PEG grafting and hydrolysis conditions but none of them gave a positive result.

The solid-phase deprotection strategy adopted in this synthetic scheme resulted in complexity that caused difficulty in understanding the reaction mechanism and kinetics such as the actual molar ratio of reagents, extent of deprotection and complete removal of the reagents and side products. Use of a PEG derivative without protecting its ω-carboxy terminal with this scheme was not viable, as it could lead to further complexity in the reaction due to a competing reaction between the two similar functional groups at the two terminals.

3.2.1.3 β-alanine ester as a cleavable linker

In seeking a new synthetic strategy using a deprotected ω-carboxy terminated PEG derivative before grafting onto a solid matrix, the hydrolysable nature of the β-alanine ester was noted (Wilchek and Miron 1987). Tateyama (1999) reported the release of Heyns rearranged sugar analogs (acetol and ribulose) from their PEG support by the alkaline hydrolysis of β-alanine ester linkage and it is now receiving more attention as a useful cleavable linker for controlled drug delivery systems (Pasut et al. 2008).

A detailed kinetic study of the hydrolysis of β-alanine ester linkage using PEG-acyclovir conjugate (Zacchigna et al. 2002) showed nearly 65% hydrolysis at pH 1.2, 9% at pH 7.4 and it remained almost stable at pH 5.5 at 37°C after 7 hour. The detailed kinetic profiles of hydrolysis at various pH values are given in Figure 3-1.

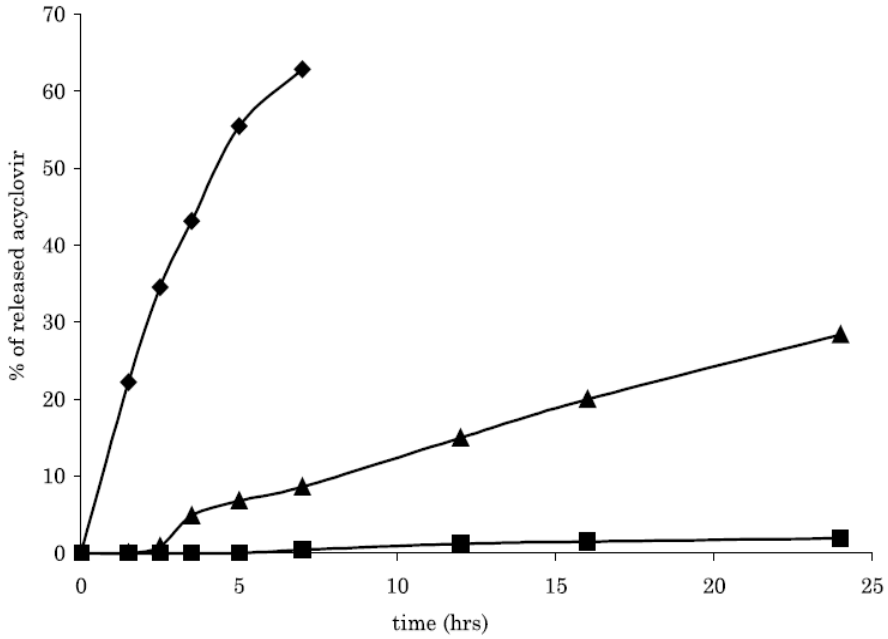


Figure 3-1: Release of acyclovir by hydrolysis of β-alanine ester linkage at 37°C from PEG-acyclovire$_2$. ♦- pH 1.2, 0.2 M (HCl, NaCl and glycine), ▲- pH 7.4, 0.1 M phosphate and ■- pH 5.5, 0.2 M, phosphate/citrate (Zacchigna et al. 2002).

These observations regarding the labile nature of β-alanine ester group, along with the availability of different functional groups at the two terminals (NH_2 group at the β-alanine terminal and COOH group at the far end of PEG), became the central basis of the new synthetic methodology for the proposed solid-phase PEGylation system.

3.2.1.4 Preparation of ω-carboxy PEG derivative

Selective monocarboxylation of PEG was very challenging, as it was very difficult to control the extent of carboxylation due to the availability of the reactive hydroxyl terminus at each end of the PEG. Efficient monocarboxylation of PEG was first reported by Harris and Kozlowski (1997), in which they first converted the PEG to a mono-nitrile derivative by treating with a stoichiometric molar amount of acrylonitrile under

alkaline conditions, followed by hydrolysing with excess sulphuric acid to its carboxyl analogue. Even though various other methodologies were reported, such as use of ethyl bromoacetate (Zalipsky and Barany 1990; Kao et al. 2005), ethyl isocyanoacetate (Zalipsky et al. 2001) and chloroacetic acid (Brock et al. 2004), the method by Harris and Kozlowski is more reliable and reproducible and it was adopted for the preparation of α- hydroxy-ω-carboxy PEG derivative.

3.2.1.5 Protection of the monocarboxy PEG terminal

The ω-carboxyl terminal prepared should be protected from other, unwanted cross reactions while modifying the α-terminal and ensure its availability during protein conjugation. Various available common carboxylic protection methodologies include conversion into esters, orthoesters, oxazoles, amides or hydrazides (Greene and Wuts 1991). However, the protection of carboxylic group through esterification was a preferable choice, as both the esterification and its deprotection can be performed under moderate conditions without affecting other functionalities present at the further end of the PEG derivative.

Esterification using lower aliphatic alcohols such as methanol and ethanol was not viable in this case, as their removal by aqueous acidic or alkaline hydrolysis could result in a substantial cleavage of the β-alanine ester linkage at the other terminal (Figure 3-1) and this emphasized the requirement for an anhydrous deprotection method.

Mainly two different protection-deprotection methodologies, allyl and benzyl methods, were developed, each characterised by particular advantages and disadvantages. The allyl method involves utilizing a soluble palladium catalyst for deprotection, with the advantage of

extending this to both solution-phase and solid-phase reactions, whereas the benzyl method involves deprotection using an insoluble palladium catalyst which can be applied only to solution-phase reactions.

3.2.1.6. Allyl protection and deprotection

Allyl protection of carboxyl group is a very efficient and frequently used method due to the stability of allyl esters under strongly acidic and basic conditions and the availability of very specific deprotection methods (Kunz and Waldmann 1984; Schelhaas and Waldmann 1996; Yu et al. 1998).

The well-established dicyclohexyl carbodiimide (DCC)-dimethylamino pyridine (DMAP) mediated esterification (Steglich esterification) method was adopted for the esterification of carboxylated PEG with allyl alcohol (Neises and Steglich 1978; Balas et al. 1989; Jarowicki and Kocienski 2001). Due to the very high efficiency and yield of this reaction, the same method was followed for all types of esterifications used in the current research.

The mechanism of the DCC-DMAP catalysed esterification (Smith et al. 1958; Smith and March 2000), illustrated in Figure 3-2, involves the formation of a pseudo-anhydride like N-acylurea intermediate (Step1) between the carboxylic acid and DCC. This intermediate reacts readily with the alcohol to form the ester, along with dicyclohexylurea (DHU) as a by-product (Step 2). Presence of a tertiary base like DMAP acts as an acyl transfer agent (Step 3), by forming an active amide intermediate with the displacement of DHU, which reacts readily with alcohol and accelerates the formation of the ester.

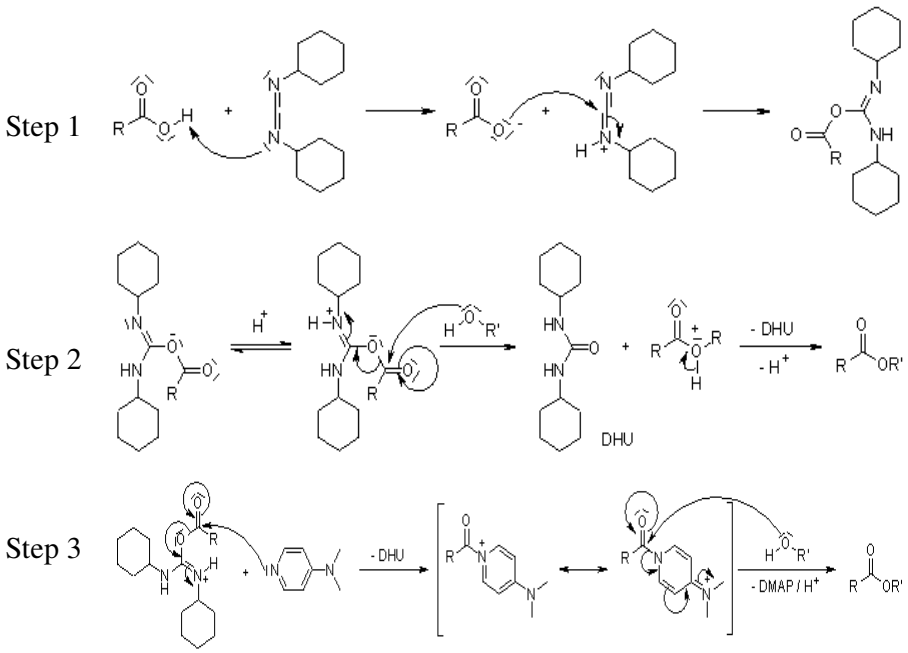
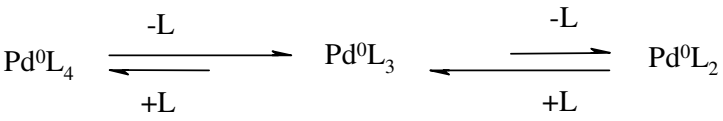
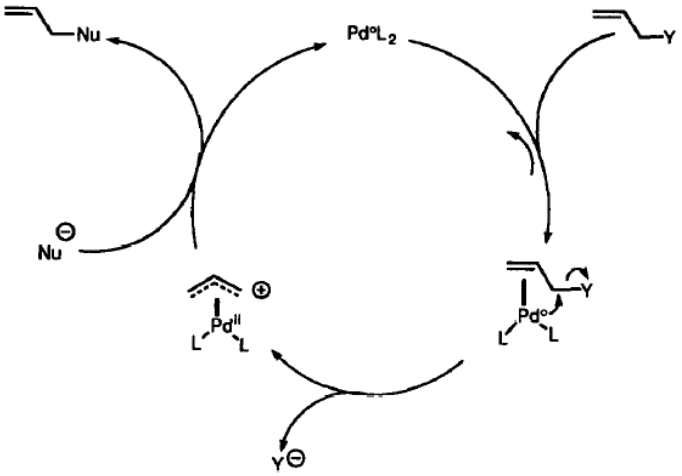


Figure 3-2: Mechanism of esterification catalysed by DCC and DMAP (Smith et al. 1958)

The most commonly used allyl deprotection method involves the catalytic transfer of allyl entity to a suitable nucleophilic scavenger by a zerovalent co-ordinately saturated palladium complex, preferably tetrakis (triphenylphosphine) palladium(0) ($Pd(PPh_3)_4$). The mechanism of allyl deprotection involves an *in situ* ligand dissociation from the palladium complex (Step 1, Figure 3-3) followed by the formation of a π-allyl palladium complex by cleaving off from the carboxyl group, with an increase in the oxidation state of palladium from zero to two. The nucleophilic allylic scavengers present in the reaction media react directly at the allylic carbon, resulting in the reductive elimination of the allyl group and the restoration of the palladium fragment to its initial zerovalent oxidation state (Step 2, Figure 3-3) (Guibe 1998).



Step 1. Ligand dissociation



Step 2. Formation of π-allyl palladium complex and subsequent allyl removal

Figure 3-3: Mechanism of allyl deprotection through catalytic palladium π-allyl methodology (Y = PEGCOO) (Guibe 1998).

A large variety of nucleophilic species, such as oxygen (potassium 2-ethylhexanoate), nitrogen (N-methyl aniline), carbon (dimedone, N,N-dimethyl barbituric acid), sulphur (2-thiobenzoic acid) and silanated (trimethylsilylazide) nucleophiles were cited as allyl scavengers. A brief summary of nucleophiles which were specifically used in this project are summarised in Table 3-2.

Table 3-2: Various nucleophiles used for the deallylation of ω-allyl PEG derivative

Nucleophile	Nature	Reference
Phenylsilane	Silanated, neutral	(Dessolin et al. 1995; Thieriet et al. 1997; Thieriet et al. 2000; Tulla-Puche and Barany 2004; Brunsveld et al. 2006)
N-methyl morpholine	Nitrogen, basic	(Albericio and Kates 1994; Lee et al. 1996; Flouzat et al. 1997; Lohse and Felber 1998; Napolitano et al. 2001; Zhang et al. 2007)
Pyrrolidine	Nitrogen, basic	(Juteau et al. 1997; Curt A. Dvorak 2000; Lienard et al. 2007)

The reaction requires very strict anhydrous conditions, as the palladium catalyst and the π-allyl palladium complex are highly susceptible to atmospheric moisture and can lead to the formation of undesired side products.

3.2.1.7 Benzyl protection and deprotection

Benzyl protection for the PEG carboxyl derivative was formulated as an alternative to allyl protection, which is characterised by the use of a non-soluble palladium catalyst for deprotection. Benzyl esters and their substituents are important protecting functional groups for carboxylic acids, due to their ease of esterification and relative stability under acidic and basic conditions. The benzyl protecting group can be quantitatively

cleaved by catalytic hydrogenolysis using a palladium on charcoal catalyst under very mild reaction conditions (Anantharamaiah and Sivanandaiah 1977; Greene and Wuts 1991). Characteristic properties of the benzyl group, such as state of hybridization, high reactivity of the benzyl carbon and the electronic interaction of the neighbouring phenyl group result in the formation of a π-benzyl intermediate with palladium, which is favoured by the electronegative carboxyl leaving group. Hydrogen acts as the primary displacing agent to facilitate the leaving of the carboxyl nucleophile from this π-benzyl intermediate, resulting in the formation of free carboxylic acids (Khan et al. 1967).

3.2.1.8 β-alanine esterification of the PEG derivative

The choice and requirements of β-alanine ester as the cleavable linker were explained previously in Section 3.2.1.3. As mentioned in Section 3.2.1.6, DCC-DMAP catalysed esterification was a suitable technique to link Boc-β-alanine on the hydroxy terminal of the carboxy protected PEG derivative. However, a very careful selection of the Boc deprotection method was necessary due to the presence of a labile ester linkage within the molecule. Various deprotection methods were available, such as TFA/H_2O/DCM (Park et al. 1996; Ripka et al. 1998), TFA/DCM (Kim et al. 1996; Paranjpe et al. 2004), concentrated H_2SO_4/DCM (Strazzolini et al. 2005), 10% H_2SO_4/Dioxane (Houghten et al. 1986) and 4 M HCl/Dioxane (Schneider and Kazmaier 1998; Atiq ur et al. 1999; Kono et al. 2000). Selection of a suitable deprotection reagent was achieved based on experimental results depending on the relative stability of the alanine ester linkage in the PEG derivative.

3.2.2 PEG derivative for hydrolysis kinetic studies

The overall objectives of the proposed solid-phase protein PEGylation method include the grafting of a functionally modified PEG derivative onto a suitable solid matrix, followed by cleaving off the conjugated PEG-protein derivative under mild conditions without denaturing the protein in a commercially acceptable quantity and quality. Due to the very high cost of protein, associated with its high probability of contamination and degradation, optimization of these parameters were planned using similar conditions but in the absence of protein.

Accurate and reproducible quantification of PEG on the solid matrix before and after hydrolysis was a significant challenge throughout the process development and there is still a very good scope for improvement in establishing a suitable methodology for this. Even though the gain in weight of the solid matrix after PEG conjugation provides an approximate estimation of the amount of PEG for establishing grafting parameters, a method to quantify the amount of PEG cleaved off the matrix is necessary to establish an optimised hydrolysis condition.

Quantification of PEG using UV estimation by complexing with ammonium ferrothiocyanate (Nag et al. 1996; Li et al. 2003; Gong et al. 2007), barium chloride-iodine (Childs 1975; Sims and Snape 1980; Gong et al. 2007) and Nessler's reagent (Ingham and Ling 1978; Harris et al. 1984) were tried. Even though very accurate and reproducible standard curves could be generated by PEG standard solutions alone, all of these procedures failed to produce a consistent, reproducible result in a heterogeneous system with PEG, NHS, protein and hydrolysing buffers, which constitute the actual reaction conditions, due to the interference in the UV estimation arising from their individual absorptions.

Use of a suitable chromatographic method such as SEC to separate the PEG from this heterogeneous reaction mixture and its quantification was a choice but was constrained by the lack of absorption of pure PEG in the UV region (Devineni et al. 2002). The optical activity of benzyl chromophores (Nomori et al. 1975) is well known and introduction of this chromophores into PEG provides a useful tool for monitoring the molecule by UV absorption and thereby its quantification using SEC.

3.2.3 PEG derivative for solution-phase protein conjugation

Solution-phase protein conjugation of the synthetic PEG derivative was a requirement for comparing the performance between the two phase conjugation methodologies and to help with the interpretation of experimental results and hypothesis. A monocarboxy-PEG derivative was conjugated to a selective functional group of protein with a specific bioconjugate technique and was characterised by using SEC and SDS–PAGE gel electrophoresis.

NHS ester is the most commonly used amine reactive species used in conjugation chemistry. This preferentially reacts with the primary amine group (N-based nucleophiles) present in the N-terminus and side chains of lysine residues in protein and peptides (Hermanson 1996). Because the present research utilizes Cytochrome c as a model protein (Section 2.6.1), protein conjugation with NHS activated PEG derivative was selected as the main bioconjugation technique and was adopted for both solution-phase and solid-phase protein conjugations.

The proposed synthetic scheme for solution-phase protein PEGylation involves the preparation of NHS ester of the PEG carboxyl derivative

under anhydrous conditions (Harris and Kozlowski 1997) followed by its conjugation with Cytochrome c in PBS buffer (Fee 2007).

3.3 Materials

PEGs with molecular weights 2000 and 4000 Da (Sigma-Aldrich, St. Louis, MO, USA) were used for modifications. Cytochrome c from bovine heart (Sigma-Aldrich) was used as the model protein for protein conjugation. Other reagents used for preparing heterobifunctional PEGs are summarised in Table 3-3.

Table 3-3: List of reagents used for preparing PEG derivatives

Chemical	Abbreviation	Manufacturer/ Supplier
Acetic acid	AcOH	Ajax Finechem Ltd, Auckland, New Zealand
Acrylonitrile		Sigma-Aldrich
Allyl alcohol		Sigma-Aldrich
Benzyl alcohol		Sigma-Aldrich
Benzylamine		Sigma-Aldrich
Boc-β-alanine	Boc-β-Ala-OH	Sigma-Aldrich
Celite		Ajax Finechem Ltd
Conc. Sulphuric acid	H_2SO_4	Ajax Finechem Ltd
4-(Dimethylamino)pyridine	DMAP	Sigma-Aldrich
Hydrochloric acid	HCl	Ajax Finechem Ltd
N,N′-Dicyclohexylcarbodiimide	DCC	Sigma-Aldrich
N,N-Diisopropylethylamine	DIEA	Sigma-Aldrich

Table 3-3 continued

N-Methylmorpholine	NMM	Sigma-Aldrich
10 % Palladium on charcoal	Pd/C	Johnson Matthey, London, UK
Potassium hydroxide	KOH	Merck, Darmstadt**,** Germany
Tetrakis(triphenylphosphine) palladium(0)	$Pd(PPh_3)_4$	Sigma-Aldrich

Various solvents used and their drying conditions are given in Table 3-4.

Table 3-4: Solvents used and their drying conditions

Solvent (Abbreviation)	**Manufacturer/ Supplier**	**Drying and storage conditions**
Dichloromethane (DCM)	Merck	Refluxed over calcium hydride and constant boiling fractions were collected. Stored over 4 Å molecular sieves under N_2.
Diethyl ether	Ajax Finechem Ltd	Anhydrous diethyl ether directly procured from the supplier and used as such.
Dimethyl formamide (DMF)	Sigma-Aldrich	Anhydrous DMF procured from Sigma-Aldrich and stored over 4 Å molecular sieves under N_2.
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich	Anhydrous DMSO procured from Sigma-Aldrich and stored over 4 Å molecular sieves under N_2.

Table 3-4 continued

1, 4-Dioxane	Sigma-Aldrich	Anhydrous 1,4-Dioxane procured from Sigma-Aldrich and stored over 4 Å molecular sieves under N_2.
Ethanol (EtOH)	Ajax Finechem Ltd	Used as received
Methanol (MeOH)	Ajax Finechem Ltd	Double distilled from magnesium metal. Constant boiling fraction were collected and stored over 4 Å molecular sieves.

10 mM PBS buffer was used for PEG protein conjugation and also for SEC analysis. PBS buffer was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 mL ultrapure water, adjusted the pH to 7.4 with HCl and finally added extra ultrapure water to make up to the 1 L (Zhang et al. 2006).

3.4 Analytical methods

3.4.1 ^{1}H NMR

^{1}H NMR spectra were used for the characterization of all PEG intermediates and for reaction monitoring in some cases. NMR spectra were recorded at 23°C on a Varian Unity INOVA-500 spectrometer operating at 500 MHz. Chemical shifts in this thesis are described in parts per million (ppm) on the δ scale and $CDCl_3$ was used as the solvent (referenced to C**H**Cl_3 at δ_H approximately 7.26 ppm).

3.4.2 Ninhydrin test detection of primary amine on PEG-alanine derivative

The ninhydrin test was performed to confirm the primary amine terminal (Kaiser et al. 1970) obtained by deprotection of Boc-β-alanine PEG derivative. A stock solution was prepared by dissolving 0.2 g of Ninhydrin in 50 mL DI water under stirring. About 2 mg of sample (or a small drop if for the samples was in solution) was added to 1 mL of the above prepared solution and heated to boil for about 30 seconds. A blue or blue violet colouration confirmed the presence of α–amino groups.

3.4.3 AKTA Explorer

SEC was used extensively to monitor the PEG-protein conjugation as well as the kinetic studies of grafted PEG from the solid matrix. Samples were analysed using a Superdex 200 HR 10/30 size exclusion column (GE Healthcare Technologies, formerly Amersham Biosciences, Uppsala, Sweden) connected to an AKTAexplorer 10XT (GE Healthcare Technologies). 10 mM PBS buffer was used as the running buffer for PEGylated protein samples and the column was first equilibrated with the running buffer before introduction of the sample. Various components present in the samples were separated based on their molecular sizes and their elution profiles were monitored by following UV absorbances at 215 and 280 nm.

For quantification of the hydrolysed PEG during the kinetics studies, 10% methanol in ultrapure water was used as the running buffer to avoid the clogging of column while keeping all other parameters constant.

3.4.4 SDS-PAGE gel electrophoresis

The sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) method is a commonly used analytical technique to separate proteins according to their molecular weights. This analytical tool was used as a supplementary method to characterise the PEGylated protein species and to determine their degree of PEG conjugation.

1–10 µL of each samples were mixed with 5 µL of 4X NuPAGE® LDS sample buffer and 2 µL of NuPAGE® sample reducing agent and heat denatured at 95–100°C for 5 minutes. Samples were run through a 12% gradient Bis-Tris NuPAGE® polyacrylamide gel and after the completion of the run the gel was stained for 30–60 minutes in staining solution (0.125% Coomassie Brilliant blue, 10% acetic acid and 50% methanol), then destained overnight with 10% acetic acid and 50% methanol. The gels were scanned using Stratagene Cheme Genius 2.

3.5 Synthetic procedures and characterizations

PEGs with two different molecular weights 2000 and 4000 Da were used for modifications, following the same synthetic schemes with the same molar ratios and reaction parameters. All synthetic strategies were adopted based on similar functional transformations or preparation of similar PEG derivatives as reviewed in Section 3.2, with some changes in the parameters and molar ratios where necessary.

3.5.1 PEG derivative for solid-phase protein conjugation

The heterobifunctional PEG derivative, α-(β-alanine)-ω-carboxy PEG derivative (**6**) was prepared by following two different schemes, namely

Scheme-1 (Figure 3-4), following the allyl protection-deprotection, and Scheme-2 (Figure 3-5), following the benzyl protection-deprotection methods. All experimental procedures were explained based on PEG 4000 Da for better understanding of reagent quantities and the same molar ratios and parameters were followed for PEG 2000 Da derivatization.

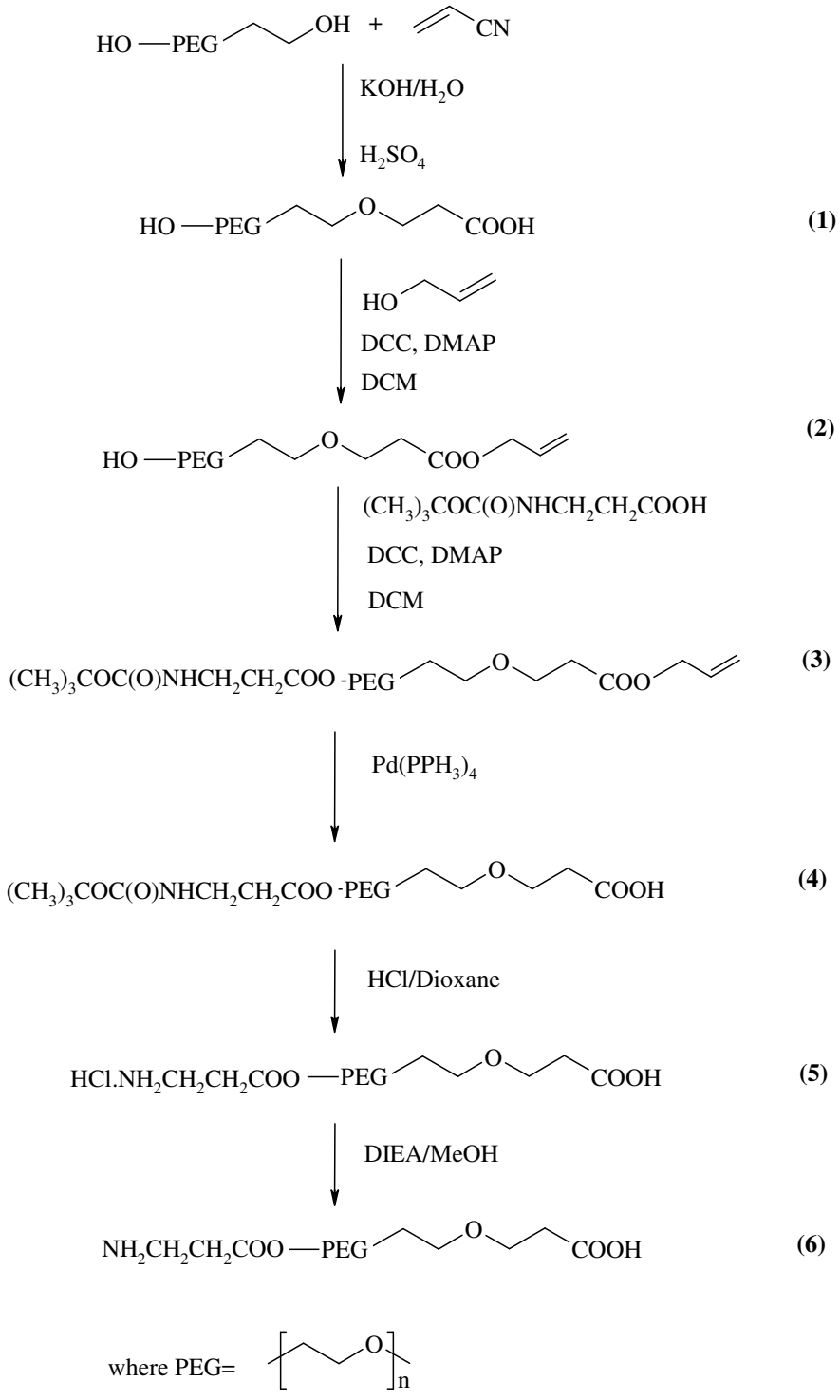


Figure 3-4: Scheme-1 Using allyl protection

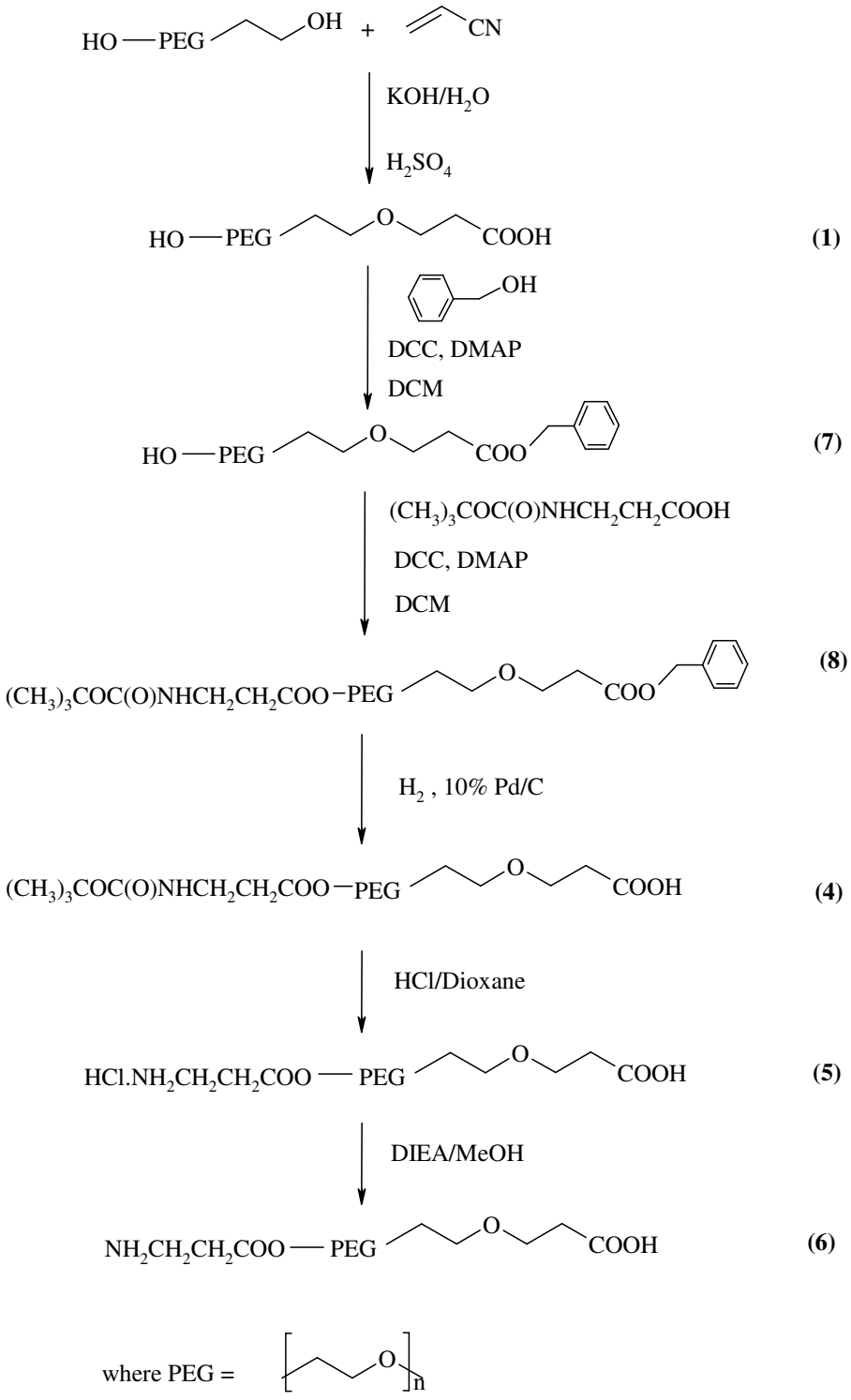


Figure 3-5: Scheme-2 Using benzyl protection

3.5.1.1 Preparation of α-hydroxy-ω-carboxy PEG (4000) derivative (1)

PEG (average MW 4000 Da, 20.0 g, 5 mmol) and KOH (0.37 g, 5.75 mmol, 1.15 molar eq.) were dissolved in DI water (25 mL) and cooled to 0°C under stirring. Acrylonitrile (0.34 g, 6.25 mmol, 1.25 molar eq.) was charged slowly over a period of half an hour and stirring was continued for 4 hour. Concentrated sulphuric acid (26.2 g, 262 mmol, 52.4 molar eq.) was added and the mixture was maintained at 95–100°C for 5 hour. After cooling to room temperature, the reaction mixture was quenched into saturated sodium chloride solution (200 mL) and the product was extracted using DCM (4 x 100 mL). The pooled DCM extracts were dehydrated using anhydrous sodium sulphate and concentrated under vacuum to isolate the crude product.

The crude product was then redissolved in DCM (20 mL, 1 vol.) and diethyl ether (400 mL, 20 vol.) was charged to precipitate the PEG derivative. After stirring for one hour to ensure the complete precipitation, the product was filtered and washed with diethyl ether (2 x 20 mL, 2 x 1 vol.). Dried the product under vacuum at 25°C (isolated quantity 17.8 g) and was confirmed from its NMR spectra. ^{1}H NMR ($CDCl_3$): -O-CH_2-C**H**$_2$-COOH (t, δ 2.553 ppm), -O-C**H**$_2$-CH_2-COOH (t, δ 4.186 ppm) and PEG back bone (-C**H**$_2$C**H**$_2$-O-)$_n$-) (δ 3.648 ppm).

3.5.1.2 Preparation of α-hydroxy-ω-allyl PEG (4000) derivative (2)

To a clear solution of intermediate **1** (17.0 g, 4.25 mmol) in anhydrous DCM (136 mL), allyl alcohol (0.37 g, 6.37 mmol, 1.5 molar eq.) was charged under N_2 atmosphere and the mixture was cooled to 0°C under stirring. A solution of DCC (2.21 g, 10.62 mmol, 2.5 molar eq.) and

DMAP (0.13 g, 1.06 mmol, 0.25 molar eq.) was prepared in DCM (34 mL) and added to the reaction mass over a period of half an hour. Cooling was removed after one hour and continued the stirring overnight at room temperature. The dicyclohexylurea by-product was removed by filtration and the clear filtrate concentrated under vacuum in rotary evaporator to get the crude product. The product was crystallized using DCM (17 mL)-diethyl ether (340 mL) mixture, as mentioned in the previous step and dried under vacuum at 25°C.

Isolated yield 16.4 g. ^{1}H NMR ($CDCl_3$): $\mathbf{H}_2C$=CH-CH_2- (dd, δ 5.163, 5.184 and 5.243, 5.277 ppm), H_2C=C**H**-CH_2- (m, δ 5.849 ppm), H_2C=CH-C$\mathbf{H}_2$- (d, δ 4.533, 4.545 ppm), -C$\mathbf{H}_2$-COO- (t, δ 2.556 ppm) and PEG back bone (δ 3.586 ppm).

3.5.1.3 Preparation of α-(Boc-β-alanine)-ω-allyl PEG (4000) derivative (3)

Intermediate **2** obtained from the previous step (16.2 g, 4.05 mmol) and Boc-β-alanine (0.97 g, 5.06 mmol, 1.25 molar eq.) were mixed together in anhydrous DCM (130 mL) under N_2 and stirred to get a clear solution. After cooling the reaction mass to 0°C, slowly charged a solution of DCC (2.11 g, 10.12 mmol, 2.5 molar eq.) and DMAP (0.12 g, 1.01 mmol, 0.25 molar eq.) in DCM (32 mL) over a period of half an hour. Cooling was removed after one hour and continued the stirring overnight. The DCC-urea by-product was filtered off and the clear filtrate was concentrated under vacuum in a rotary evaporator. Crude product was then crystallized using DCM (16 mL)-diethyl ether (320 mL) mixture, as in Section 3.5.1.2 and was characterised using ^{1}H NMR spectrum.

Isolated yield 16.24 g. ^{1}H NMR ($CDCl_3$) (Figure 3-6): ($C\mathbf{H}_3$)$_3$C-O-CO- (s, δ 1.399 ppm), -NH-C$\mathbf{H}_2$-CH_2-CO-O- (t, δ 3.355 ppm), -NH-CH_2-C$\mathbf{H}_2$-CO-O- (t, δ 2.519 ppm), -CO-O-C$\mathbf{H}_2$-CH_2-O- (t, δ 4.233 ppm), -CO-O-CH_2-C$\mathbf{H}_2$-O- (t, δ 3.467 ppm), -O-CH_2-C$\mathbf{H}_2$-CO-O- (t, δ 2.596 ppm), -O-C$\mathbf{H}_2$-CH=CH_2 (d, δ 4.558, 4.570 ppm), -O-CH_2-C$\mathbf{H}$=CH_2 (m, δ 5.886 ppm), -O-CH_2-CH=C$\mathbf{H}_2$ (dd, δ 5.187, 5.208 and 5.269, 5.302 ppm) and PEG back bone (δ 3.611 ppm).

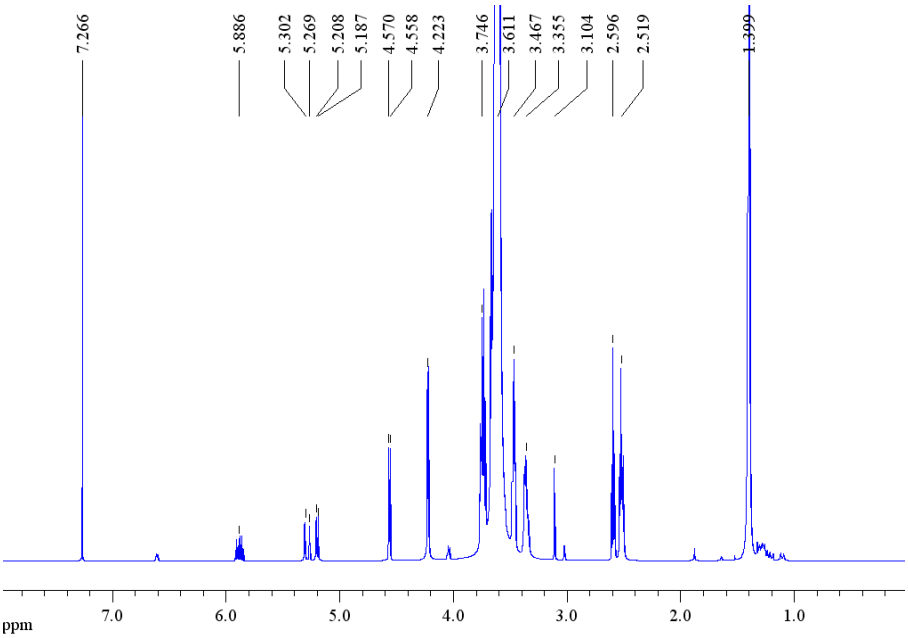


Figure 3-6: ^{1}H NMR of α-(Boc-β-alanine)-ω-allyl PEG (4000) derivative (**3**)

3.5.1.4 Deallylation and preparation of α-(Boc-β-alanine)-ω-carboxy PEG (4000) derivative (4)

Deallylation was performed with $Pd(PPh_3)_4$. Experiments were conducted using various allyl scavengers such as phenylsilane, N-methylmorpholine (NMM) and pyrrolidine and all these methods resulted in specific

removal of the allyl protection very efficiently and quantitatively, without cleaving the other terminal ester linkage.

In order to confirm the specificity of this deprotection and compatibility with the ester group at the α terminal of the bifunctional PEG derivative, a kinetic study of the allylic deprotection was performed with $Pd(PPh_3)_4$ and NMM and phenylsilane as allyl scavengers. For this, a PEG derivative was prepared with 2-butyl ester group one terminus and allyl group at the other end (intermediate **10**) following the Scheme-3 (Figure 3-7).

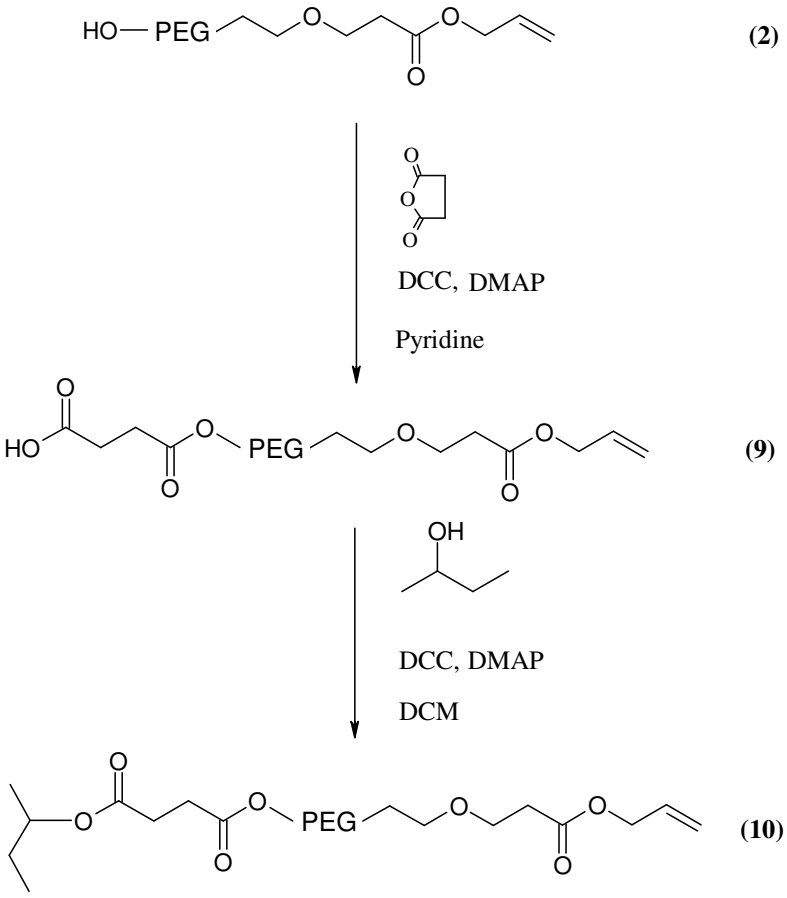


Figure 3-7: Scheme-3 Preparation of 2-butyl ester for deallylation reaction monitoring

α-hydroxy-ω-allyl PEG derivative (intermediate **2**, Section 3.5.1.2) was converted into its α-succinyl derivative **(9)** by treating with succinic anhydride (5 molar eq.) in presence of DMAP (0.5 molar eq.) in anhydrous pyridine (1.5 vol.) (Krepinsky et al. 1994). The product was crystallized using DCM-diethyl ether mixture and characterised as the DMAP salt of the succinic acid PEG derivative from its NMR spectrum.

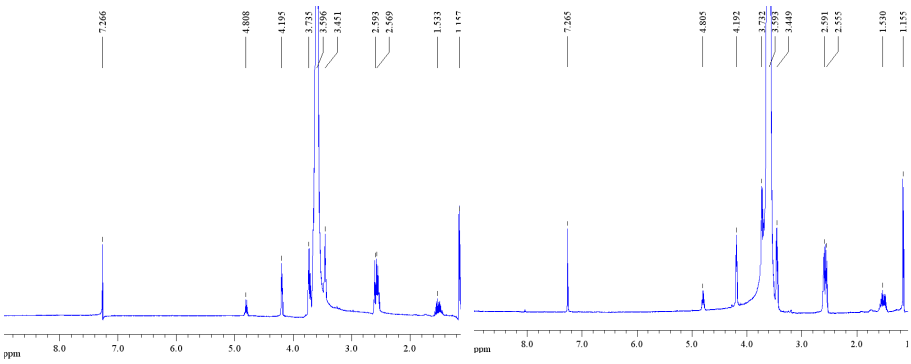
^{1}H NMR in $CDCl_3$: $\mathbf{H}_2C$=CH-CH_2- (dd, δ 5.163, 5.184 and 5.240, 5.274 ppm), H_2C=C**H**-CH_2- (m, δ 5.847 ppm), H_2C=CH-C$\mathbf{H}_2$- (d, δ 4.532, 4.543 ppm), -O-CO-C$\mathbf{H}_2$-CH_2-O- (t, δ 4.191 ppm), -CO-C$\mathbf{H}_2$C$\mathbf{H}_2$-CO- (succinyl) and O-CH_2-C$\mathbf{H}_2$-CO- (m, δ 2.570 ppm), PEG backbone (δ 3.585 ppm), pyridine **H**s from DMAP (d, δ 6.627, 6.642 and 8.155, 8.168) and –N(C$\mathbf{H}_3$)$_2$ (s, δ 3.137 ppm).

Intermediate **9** was esterified with 2-butanol following the same esterification method using DCC and DMAP as mentioned in sections 3.5.1.2 and 3.5.1.3. to get the α-(2-butyl)-ω-allyl PEG derivative **(10)**. ^{1}H NMR ($CDCl_3$): C$\mathbf{H}_3$-CHO-CH_2-CH_3 (d, δ 1.139, 1.152 ppm), CH_3-C**H**O-CH_2-CH_3 (m, δ 4.802 ppm), CH_3-CHO-C$\mathbf{H}_2$-CH_3 (m, δ 1.527 ppm), CH_3-CHO-CH_2-C$\mathbf{H}_3$ (t, δ 0.835 ppm), $\mathbf{H}_2$C=CH-CH_2- (dd, δ 5.166, 5.188 and 5.244, 5.278 ppm), H_2C=C**H**-CH_2- (m, δ 5.865 ppm), H_2C=CH-C$\mathbf{H}_2$- (d, δ 4.536, 4.548 ppm), -CO-O-C$\mathbf{H}_2$-CH_2-O- (t, δ 4.180 ppm), -CO-C$\mathbf{H}_2$C$\mathbf{H}_2$-CO- (succinyl) (m, δ 2.587 ppm), O-CH_2-C$\mathbf{H}_2$-CO- (t, δ 2.487 ppm) and PEG backbone (δ 3.590 ppm).

3.5.1.4.1 Deallylation using phenylsilane as allyl scavenger

Deallylation with phenylsilane was performed by treating intermediate **10** with 1.2 equivalents of $Pd(PPH_3)_4$ and 24 equivalents of phenylsilane under N_2 atmosphere. Samples were withdrawn periodically after 1, 2, 4

and 12 hour and were immediately crystallised from DCM / diethyl ether mixture. The ^{1}H NMR spectra of these samples shows the completion of deallylation within 1 hour (Figure 3-8A) and butyl group remained intact with the molecule even after 12 hour (Figure 3-8B) indicating the very specificity of the reagents.

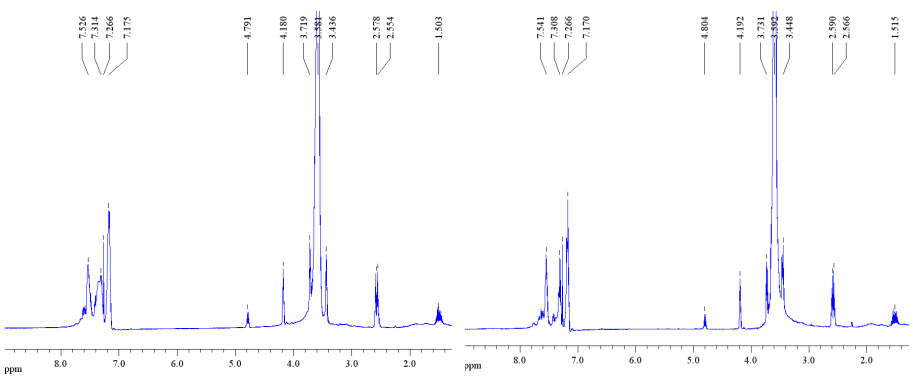


A) After 1 Hr **B)** After 12 Hr

Figure 3-8: ^{1}H NMR of deallylated PEG derivative using $Pd(PPH_3)_4$ and phenylsilane

3.5.1.4.2 Deallylation using NMM as allyl scavenger

Intermediate **10** was treated with 1.2 equivalents of $Pd(PPH_3)_4$ in a mixture of chloroform-acetic acid-NMM (37:2:1, 13 vol.) under N_2. As in the case with phenylsilane, samples were withdrawn periodically after 1, 2, 4 and 12 hour and the crystallised samples were analysed by ^{1}H NMR. The selectivity and specificity of the deallylation was exactly similar to the previous method and also the reaction was completed within 1 hour. (Figure 3-9A for 1 hour and Figure 3-9B for 12 hour sample).



A) After 1 Hr **B)** After 12 Hr

Figure 3-9: ^{1}H NMR of deallylated PEG derivative using $Pd(PPH_3)_4$ and chloroform-acetic acid-NMM

^{1}H NMR spectra of the deprotected products using $Pd(PPH_3)_4$ and chloroform-acetic acid-NMM shows presence of multiplets at δ values 7.1 to 7.5 ppm indicating the presence of triphenylphosphine impurity along with the deprotected PEG derivative. The probable reason for this is due to the oxidative coupling of chloroform with $Pd(PPH_3)_4$ resulting in formation of the complex $Pd(PPh_3)_2CHCl_3$, with displacement of two molecules of triphenylphosphine ligand (Kaliya et al. 1969). This complex is insoluble in diethyl ether and crystallized out along with the PEG derivative, imparting an yellow colouration to the product.

Based on these observations and product profile, phenylsilane was selected as the suitable allyl scavenger for further experimentation. Optimization of the process leads to a complete removal of the allyl group with 0.25 molar equivalents of $Pd(PPH_3)_4$ within 4 hour and the deallylation process was finalized as below.

Very strict moisture control was taken care during allyl deprotection and all glasswares were cleaned and dried overnight at 100°C and flushed with enough N_2 before raw material charging. α-(Boc-β-alanine)-ω-allyl PEG derivative **(3)** (15.0 g, 3.75 mmol) and $Pd(PPh_3)_4$ (1.12 g, 0.94

mmol, 0.25 eq.) were mixed in dry freshly distilled DCM (120 mL) under N_2 and stirred to get a clear solution. Prepared a solution of phenylsilane (10.0 g, 90 mmol, 24 eq.) in dry DCM (30 mL) and charged into the reaction mass within 15 minutes and stirred for 4 hour under N_2 atmosphere. Quenched the mass slowly under stirring into diethyl ether (1 L) and stirred for 1 hour to allow the complete precipitation. Filtered the mass under vacuum and washed thoroughly with diethyl ether (4 x 50 mL). Recrystallized the product second time with DCM (34 mL)-diethyl ether (340 mL) mixture. Isolated yield 13.5 g

3.5.1.5 Boc removal and preparation of α-(β-alanine)-ω-carboxy PEG (4000) derivative (6)

Removal of the Boc protecting group at the N terminal of alanine with TFA (50 molar eq.) was tried but only partial deprotection was achieved with about 55% Boc group left behind with the PEG and further reprocessing of the material was necessary to get the complete removal of the protecting group. Due to the high cost of TFA along with reprocessing of the PEG derivative to achieve complete deprotection necessitates the requirement of an easy and cost effective alternate methodology.

Use of HCl-Dioxane mixture was promising with the easy preparation of HCl gas from commercially available NaCl and conc. H_2SO_4. HCl gas thus prepared was absorbed in a known volume of dioxane and was used for Boc deprotection. Use of 4 M HCl/dioxane (30 molar eq.) resulted in removing 80% of the Boc protection within 3 hour (Figure 3-10). ^1H NMR ($CDCl_3$) : -NH_3^+ (HCl salt) (s, δ 7.930 ppm), -NH-C**H**$_2$-CH_2-CO- (t, δ 3.292 ppm), -NH-CH_2-C**H**$_2$-CO- (t, δ 2.581 ppm), -CO-O-C**H**$_2$-CH_2-

(t, δ 4.288 ppm), -CO-O-CH_2-C**H**$_2$- (t, δ 3.477 ppm), -O-CH_2-C**H**$_2$-CO-O- (t, δ 2.924 ppm) and PEG back bone (δ 3.621 ppm).

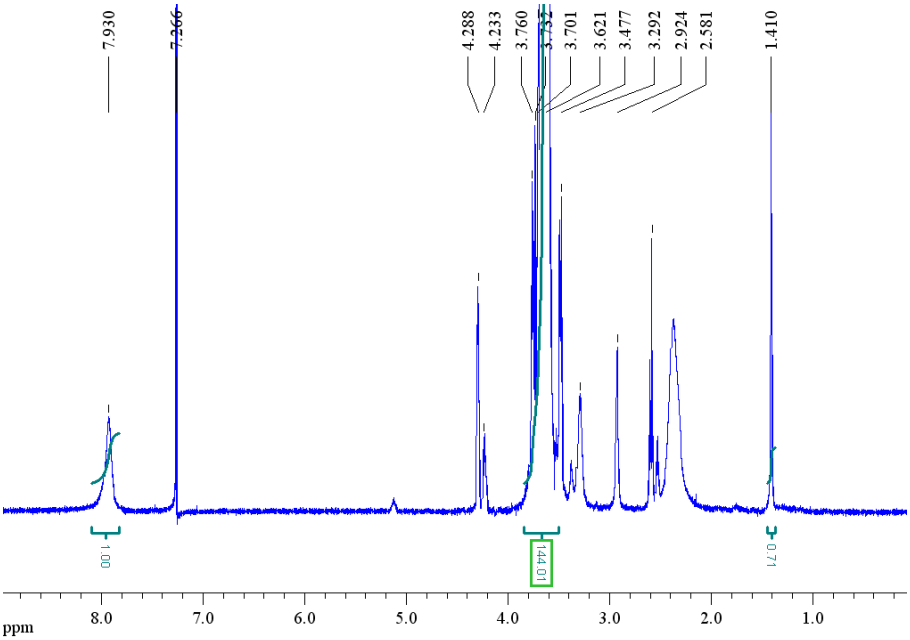


Figure 3-10: ^{1}H NMR of PEG derivative after deprotection using 30 eq. HCl (4 M HCl/dioxane)

Complete Boc removal was observed when deprotection was done with 40 eq. HCl and this molar ratio was adopted for further deprotection experiments and the process was finalised as below for the preparation of intermediate **5**.

α-(Boc-β-alanine)-ω-carboxy PEG derivative **(4)** (13.2 g, 3.3 mmol) was dissolved in DCM (132 mL) and cooled to 0°C. Slowly charged 4 M HCl/dioxane mixture (37.8 g, 132 mmol, 40 molar eq.) over a period of half an hour and maintained at that temperature for 4 hour. N_2 was carefully bubbled through the reaction mass to vent out the excess HCl gas and then concentrated the product under vacuum. The product was recrystallized using DCM (13 mL)-diethyl ether (264 mL) mixture.

Isolated yield 12.89 g.^{1}H NMR ($CDCl_3$): -NH_3^+ (HCl salt) (s, δ 7.853 ppm), -NH-C**H**$_2$-CH_2-CO- (t, δ 3.266 ppm), -NH-CH_2-C**H**$_2$-CO- (t, δ 2.548 ppm), -CO-O-C**H**$_2$-CH_2- (t, δ 4.253 ppm), -CO-O-CH_2-C**H**$_2$- (t, δ 3.452 ppm), -O-CH_2-C**H**$_2$-CO-O- (t, δ 2.892 ppm) and PEG back bone (δ 3.597 ppm).

HCl salt of the alanine-PEG derivative **(5)** (12.5 g, 3.12 mmol) was then dissolved in dry methanol (250 mL) and 10% DIEA in methanol was added drop wise till the pH is slightly basic to about pH 8.0. Stirred the mass for one hour maintaining the pH with addition of DIEA, if required, with continuous monitoring by Ninhydrin test (Section 3.4.2), till the test give persistent violet colour. The product was concentrated under vacuum to remove the methanol and product was crystallized using DCM (12.5 mL)-diethyl ether (250 mL) to get the free base (intermediate **6**). Isolated yield 11.6 g.

3.5.1.6 Preparation of α-hydroxy-ω-benzyl PEG (4000) derivative (7)

To a clear solution of α-hydroxy-ω-carboxy polyethylene glycol **(1)** (15.0 g, 3.75 mmol) in anhydrous DCM (120 mL), benzyl alcohol (0.61 g, 5.63 mmol, 1.5 molar eq.) was charged under N_2 atmosphere and the mixture was cooled to 0°C under stirring. A solution of DCC (1.95 g, 9.38 mmol, 2.5 molar eq.) and DMAP (0.12 g, 0.94 mmol, 0.25 molar eq.) in DCM (30 mL) was prepared and charged in to the reaction mass over a period of half an hour. Cooling was removed after one hour and continued the stirring overnight at room temperature.

Dicyclohexylurea by-product formed during the reaction was removed by filtering the mass over Buchner funnel and the clear filtrate was concentrated under vacuum in rotary evaporator to get the crude product.

The crude product was crystallised using DCM (30 mL)-diethyl ether (300 mL) mixture and was characterised from its ^{1}H NMR spectrum. Isolated yield 14.22 g.

^{1}H NMR ($CDCl_3$): Benzyl Ar **H**s (m, δ 7.260 ppm) and C_6H_5-C**H**$_2$- (s, δ 5.045 ppm), -O-CH_2-C**H**$_2$-CO-O- (t, δ 2.558 ppm) and PEG back bone (δ 3.553 ppm).

3.5.1.7 Preparation of α-(Boc-β-alanine)-ω-benzyl PEG (4000) derivative (8)

Benzyl protected polyethylene glycol **(7)** (14 g, 3.5 mmol) was converted into its Boc-β-alanine ester derivative **(8)** by following the same procedure described in Section 3.5.1.3. Isolated product quantity 12.45 g.

^{1}H NMR ($CDCl_3$) (Figure 3-11): (C**H**$_3$)$_3$C-O-CO- (s, δ 1.407 ppm), -NH-C**H**$_2$-CH_2-CO-O- (t, δ 3.363 ppm), -NH-CH_2-C**H**$_2$-CO-O- (t, δ 2.624 ppm), -CO-O-C**H**$_2$-CH_2-O- (t, δ 4.231 ppm), -CO-O-CH_2-C**H**$_2$-O- (t, δ 3.474 ppm), -O-CH_2-C**H**$_2$-CO-O- (t, δ 2.526 ppm) PEG back bone (δ 3.618 ppm), Benzyl Ar **H**s (m, δ 7.326 ppm) and C_6H_5-C**H**$_2$- (s, δ 5.111 ppm).

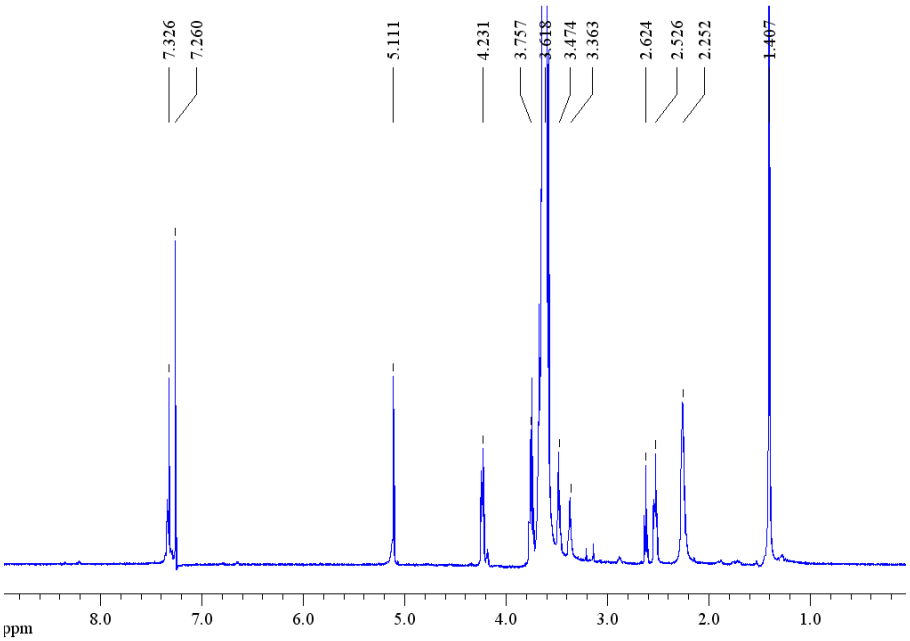


Figure 3-11: ^{1}H NMR of α-(Boc-β-alanine)-ω-benzyl PEG (4000)

3.5.1.8 Preparation of α-(Boc-β-alanine)-ω-carboxy PEG (4000) derivative (4) by debenzylation of (8)

A solution of benzyl protected PEG derivative **(8)** (12.0 g, 3 mmol) in dry methanol (120 mL) was prepared and charged 10% Pd/C (1.2 g, 10% w/w). The reaction flask was filled with hydrogen and stirred vigorously for 24 hour at room temperature. Spent catalyst was filtered off over celite and washed with methanol (2 x 12 mL). The product was concentrated under vacuum and crystallized using DCM (12 mL)-diethyl ether (240 mL) mixture. Isolated yield 10.4 g.

^{1}H NMR ($CDCl_3$): $(C\mathbf{H}_3)_3C$-O-CO- (s, δ 1.380 ppm), -NH-$C\mathbf{H}_2$-CH_2-CO-O- (t, δ 3.341 ppm), -NH-CH_2-$C\mathbf{H}_2$-CO-O- (t, δ 2.500 ppm), -CO-O-$C\mathbf{H}_2$-CH_2-O- (t, δ 4.203 ppm), -CO-O-CH_2-$C\mathbf{H}_2$-O- (t, δ 3.379 ppm), -O-CH_2-$C\mathbf{H}_2$-CO-O- (t, δ 2.540 ppm) and PEG back bone (δ 3.594 ppm).

3.5.2 Preparation of α-(β-alanine)-ω-benzyl amide PEG (4000) derivative for hydrolysis kinetic studies

α-(β-alanine)-ω-benzyl amide PEG derivative with molecular weight 4000 Da was used for the hydrolysis kinetic studies and Scheme-4 (Figure 3-12) illustrates the reaction sequence used for their preparation.

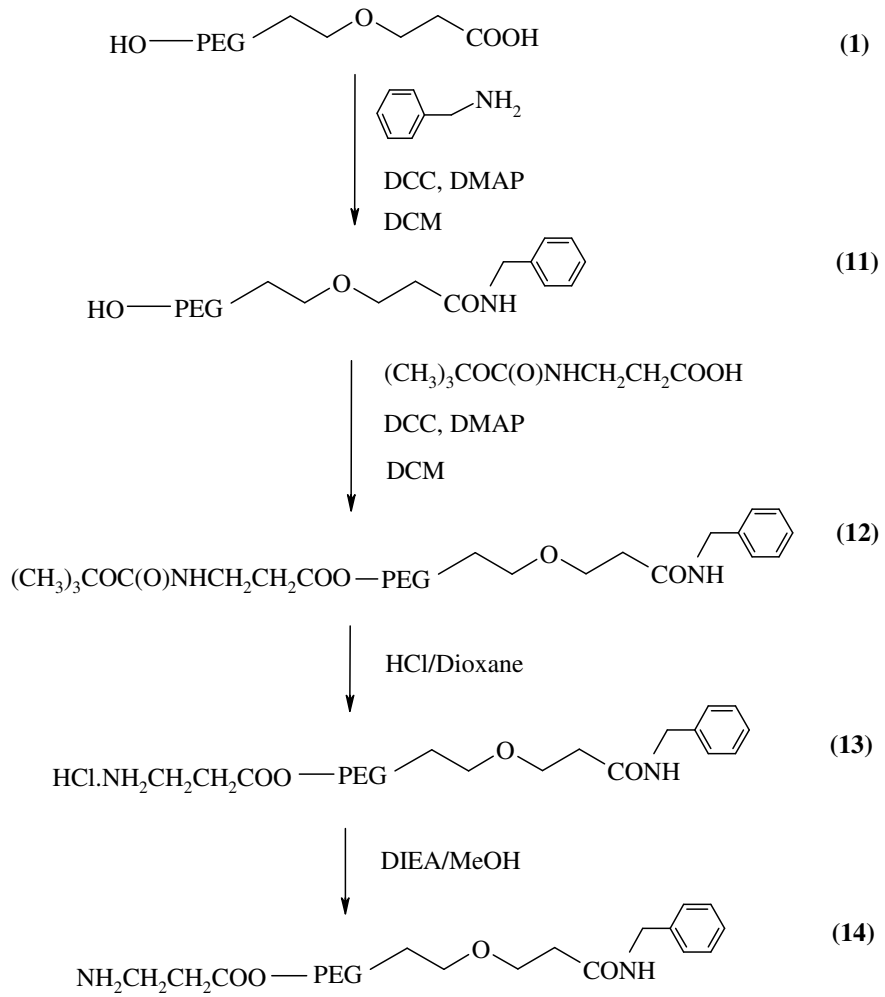


Figure 3-12: Scheme-4 Preparation of α-(β-alanine)-ω-benzyl amide PEG derivative

α-hydroxy-ω-carboxy polyethylene glycol **(1)** was converted into its benzyl amide **(11)** by reacting with benzyl amine (1.5 molar eq.) in presence of DCC (2.5 molar eq.) and DMAP (0.25 molar eq.) in anhydrous DCM (10 vol.) under N_2 atmosphere. Intermediate **11** was then esterified with Boc-β-alanine at the α-hydroxyl terminal **(12)** (refer section 3.5.1.3), the Boc group deprotected with 4 M HCl/dioxane (Section 3.5.1.5) and finally neutralized with DIEA in anhydrous methanol to produce α-(β-alanine)-ω-benzyl amide PEG derivative **(14)**.

^{1}H NMR of intermediate **14** ($CDCl_3$): NH_2-C**H**$_2$-CH_2-CO- (t, δ 3.084 ppm), NH_2-CH_2-C**H**$_2$-CO- (t, δ 2.933 ppm), -CO-O-C**H**$_2$-CH_2- (t, δ 4.248 ppm), -CO-O-CH_2-C**H**$_2$- (t, δ 3.477 ppm), -O-CH_2-C**H**$_2$-CO-O- (t, δ 2.524 ppm), PEG back bone (δ 3.534 ppm), -CO-NH-C**H**$_2$- (s, δ 2.524 ppm) and benzyl Ar **H**s (m, δ 7.221 to 7.293 ppm).

3.5.3 Solution-phase protein conjugation using PEG-N-hydroxysuccinimide (PEG-NHS) ester

As mentioned in Section 3.2.3 monocarboxy PEG was first converted into its NHS ester and then selectively conjugated through N terminal amine group present in the protein. The chemistry is illustrated in Scheme-5 (Figure 3-13) and Cytochrome c was used as the model protein for protein conjugation.

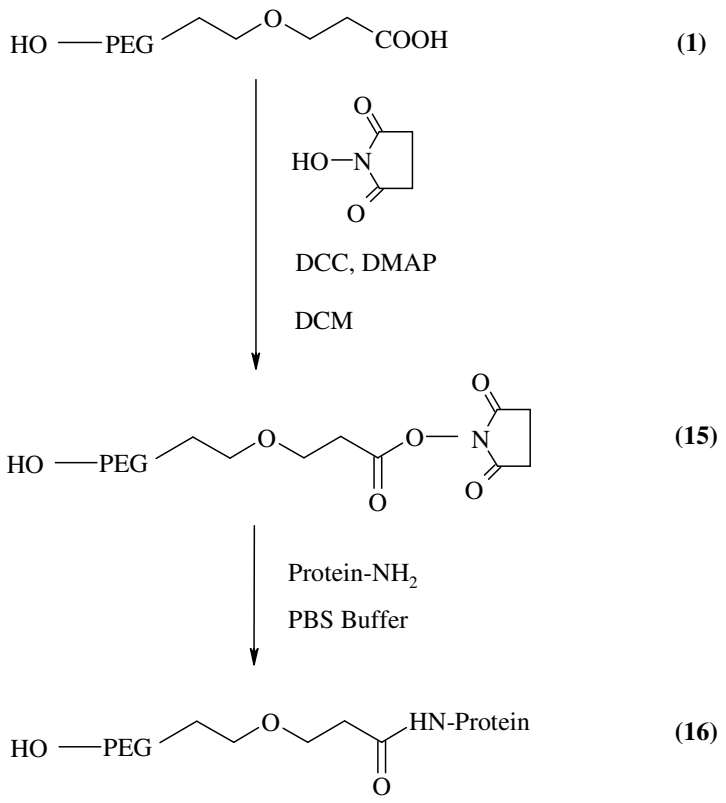


Figure 3-13: Scheme-5 Preparation of PEG-NHS and solution-phase protein conjugation

3.5.3.1 Preparation of PEG-NHS derivative (15)

To a clear solution of α-hydroxy-ω-carboxy polyethylene glycol **(1)** (1.0 g, 0.25 mmol) in anhydrous DCM (8 mL), charged NHS (0.06 g, 0.525 mmol, 2.1 molar eq.) under N_2 and cooled the mass to 0°C under stirring. To this, a solution of DCC (0.11 g, 0.525 mmol, 2.1 molar eq.) in DCM (2 mL) was added slowly and stirred the mass for 12 hour at 20°C. Precipitated urea by-product was filtered off and the clear filtrate was concentrated under vacuum. The crude product was crystallized using DCM (1 mL)-diethyl ether (20 mL) and was stored under N_2 at 4°C.

^{1}H NMR ($CDCl_3$): NHS **H**s (s, δ 2.821 ppm), -O-CH_2-C**H**$_2$-CO-O- (t, δ 2.573 ppm) and PEG back bone (δ 3.618 ppm).

3.5.3.2 Cytochrome c conjugation with PEG-NHS ester

Stock solutions of 20 mg/mL PEG-NHS ester **(15)** and 20 mg/mL Cytochrome c were prepared with 10 mM PBS buffer at pH 7.4. 5 mL of these solutions were mixed together in a 25 mL conical flask and stirred for 1 hour at 20°C. Reaction was terminated by adding a drop of 0.1 M HCl and 500 μL sample was then analysed by SEC following the method given in Section 3.4.3.

3.6 Results and discussions

3.6.1 Heterobifunctional PEG derivatives

Various heterofunctional PEG derivatives relevant to the project were synthesised and were characterized from their NMR spectra. The PEG precursor for solid-phase PEGylation, α-(β-alanine)-ω-carboxy PEG derivative (**6**), prepared following two different methods (Scheme-1 and 2, Figure 3-4 and 3-5) resulted in almost similar quality products. Highly selective and fast deallylation deprotection strategy using soluble $Pd(PPH_3)_4$ and relatively slow but selective debenzylation methodology using heterogeneous Pd/C were also provided very useful and promising synthetic strategies for preparing various PEG derivatives.

An inherent disadvantage of UV estimation arises due to lack of any UV absorbing chromophore group available with the PEG chain was overcame by derivatizing with a benzyl amide functional group (Scheme-4, Figure 3-12). This method proved very useful in quantification of PEG derivatives and was used as a useful tool in

quantifying the hydrolysed PEGs obtained from various PEG grafted solid matrices (Section 4.6.4)

3.6.2 Solution-phase protein PEGylation

The molecular weight profiles of the PEGylated Cytochrome c and other by-products were monitored by following their UV absorbances at 280 and 215 nm and are plotted in Figure 3-14 (of PEG 4000 Da) and 3-15 (of PEG 2000 Da).

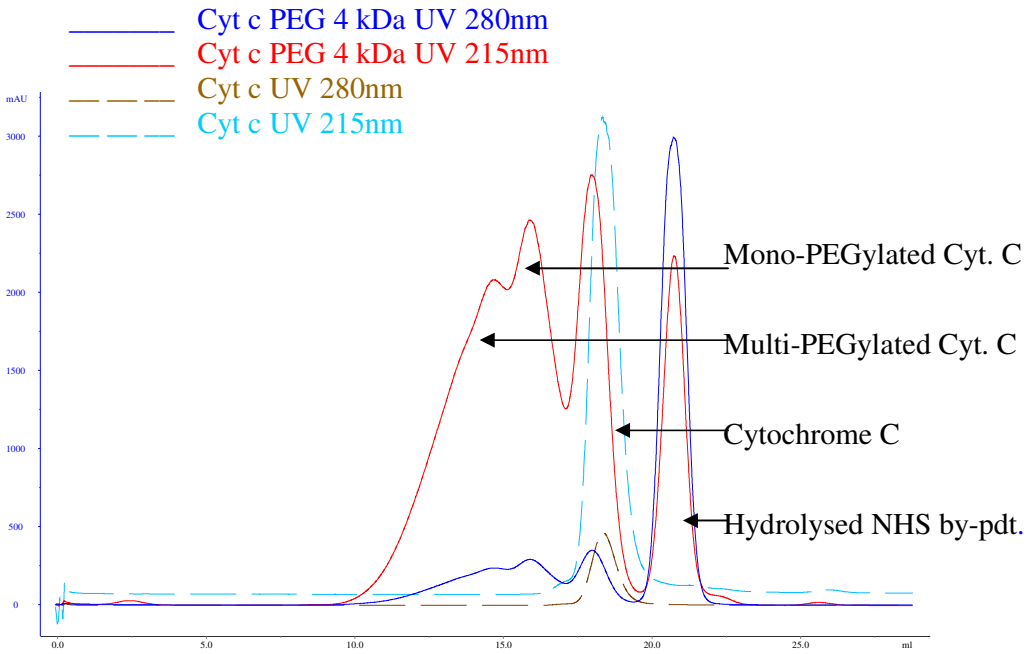


Figure 3-14: PEGylated Cytochrome c with 4000 Da PEG

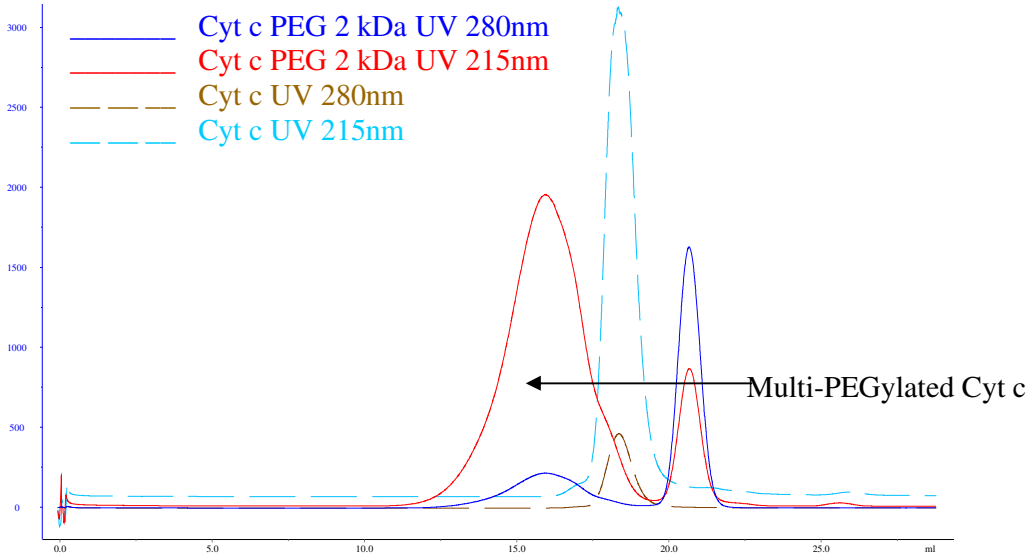


Figure 3-15: PEGylated Cytochrome c with 2000 Da PEG

The low resolution between the peaks of PEGylated products in these chromatographs are mainly due to the limitation with the efficiency of SEC to separate species with small difference in the molecular sizes. A better separation can be achieved, if these species differed in their molecular weights by about 100% (Fee and Van Alstine 2006).

The hydrodynamic radii ($R_{h,\ PEGprot}$) of the PEGylated Cytochrome c were calculated using Equation 3-1, formulated by Fee and Van Alstine (2004)

$$R_{h,PEGprot} = \frac{1}{6}\left[108R_{h,prot}^3 + 8R_{h,PEG}^3 + 12\left(81R_{h,prot}^6 + 12R_{h,prot}^3 R_{h,PEG}^3\right)^{1/2}\right]^{1/3}$$

$$+ \frac{2}{3}\frac{R_{h,PEG}^2}{\left[108R_{h,prot}^3 + 8R_{h,PEG}^3 + 12\left(81R_{h,prot}^6 + 12R_{h,prot}^3 R_{h,PEG}^3\right)^{1/2}\right]^{1/3}} + \frac{1}{3}R_{h,PEG} \quad (3\text{-}1)$$

where $R_{h,\ prot}$ is the viscosity radius (Å) of Cytochrome c, calculated using Equation 3-2 and $M_{r,\ prot}$ is its molecular weight 12,130 Da (Flatmark 1967). $R_{h,PEG,}$ the viscosity radius of the PEG used with a molecular weight $M_{r,PEG}$ and was calculated using Equation 3-3 (Fee and VanAlstine 2004).

$$R_{h,prot} \approx (0.82 \pm 0.02) M_{r,prot}^{1/3} \tag{3-2}$$

$$R_{h,PEG} = 0.1912 M_{r,PEG}^{0.559} \tag{3-3}$$

Calculated viscosity radius of Cytochrome c, PEGs and their PEGylated products are summarised in Table 3-5. The difference between the hydrodynamic radii of various PEGylated Cytochrome c species with 2000 Da PEG is very small, comparing with that of 4000 Da MW. This resulted in almost no separation of the products during their chromatographic elution in Figure 3-15.

Table 3-5: Size comparison between Cytochrome c and its various PEGylated products

MW of PEG used Da	Species	Degree of PEGylation N	Viscosity radius in Å
	Cytochrome c		18.83
	PEG		19.73
4000	mono-PEGylated	1	28.13
	di-PEGylated	2	34.62
	tri-PEGylated	3	40.52
	PEG		13.39
2000	mono-PEGylated	1	24.52
	di-PEGylated	2	28.13
	tri-PEGylated	3	31.48

The various column fractions corresponding to PEGylated species were analyzed by SDS-PAGE (12% gel, Coomassie-staining) (Section 3.4.4) and confirmed the presence of multi-PEGylated products with both 4000 Da (Figure 3-16) and 2000 Da (Figure 3-17) PEG derivatives.

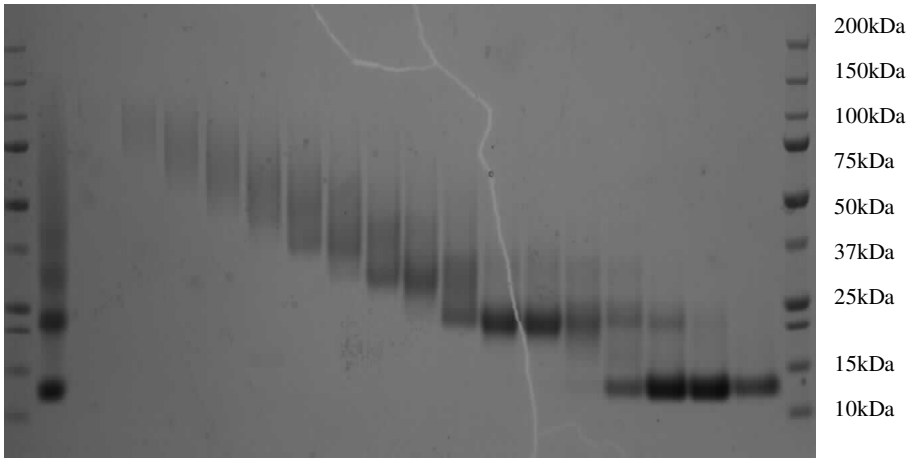


Figure 3-16: SDS-PAGE analysis of PEGylated Cytochrome c (4000 Da PEG). Line 1 & 19: Molecular weight ladder, L2: Crude PEGylated Cytochrome c (before SEC), L5 to 9: multi-PEGylated products, L10 &11: di-PEGylated product, L 13 to 15 mono-PEGylated products and L 18: Cytochrome c.

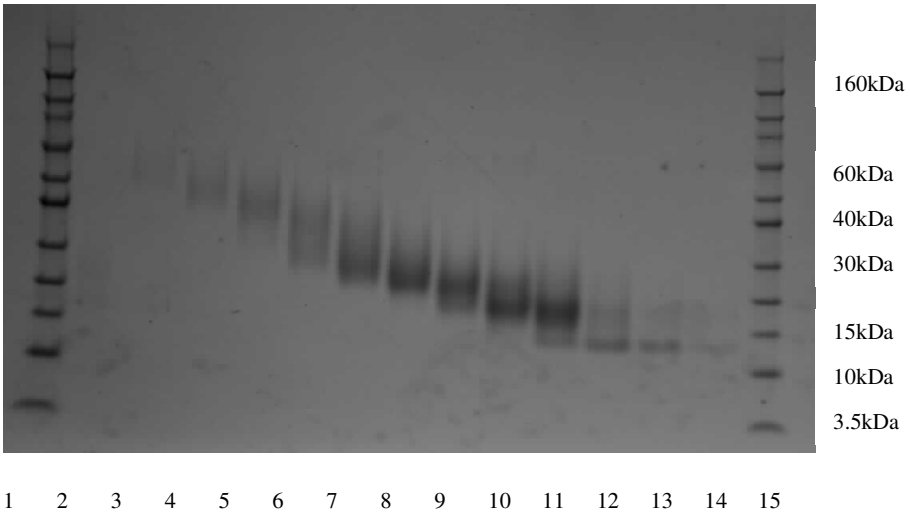


Figure 3-17: SDS-PAGE analysis of PEGylated Cytochrome c (2000 Da PEG). Line 1-2 & 15: Molecular weight ladder, L3 to 6: multi-PEGylated products, L7 to 9: di-PEGylated product, L 10 & 11: mono-PEGylated products and L 13: Cytochrome c.

Even though SDS-PAGE proved to be a very efficient and simple characterization technique for proteins, the separation was not good for PEGylated products. This phenomenon of band broadening and smearing of PEGylated products has been observed with many proteins during their SDS-PAGE analysis (Zheng et al. 2007). This is probably due to the formation of a complex between PEG and SDS, in which the polymeric PEG chains assumes a strained conformation in order to bind to the small SDS micellar cluster (Bernazzani et al. 2004). The influence of SDS concentration on this smearing effect (Odom et al. 1997) provides valuable support for this hypothesis, with a decreasing effect with a reduction in the SDS concentration.

3.7 Conclusions

Various methods were developed and optimised for the preparation heterobifunctional PEG derivatives for surface grafting, protein

conjugation and hydrolysis studies. A suitable tether for the solid-phase protein PEGylation, α-(β-alanine)-ω-carboxy PEG derivative **(6)** was synthesised and characterized.

Solution-phase protein PEGylation was performed using PEG-NHS derivative and the various PEGylated products were characterised using SEC and SDS gel electrophoresis. Hydrodynamic radii of products with various degrees of PEGylation were calculated and their elution profiles were explained based on these results.

4 Surface grafting, hydrolysis and solid-phase protein PEGylation

4.1 Introduction

This chapter provides a detailed description of the PEG grafting profile onto various modified solid matrices, as well as studies of its hydrolysis kinetics. A method for preparing the solid-phase PEGylation matrix is the main focus of this chapter and the characterization of the PEGylated protein prepared using this novel matrix is also described.

4.2 Strategy development

The choice of β-alanine ester as the cleavable linker was described earlier, in Section 3.2.1.3. The amine terminal of this derivative can be covalently grafted onto a matrix surface through a relatively stable amide linkage, if it is provided with carboxyl functional groups on the surface matrix. Due to the relatively stable nature of amide linkages in both acidic and alkaline conditions compared to ester linkages, these bonds will remain intact during hydrolytic cleaving of the alanine ester bond and thus will not interfere with the cleaved product. Keeping this in mind, carboxy modified matrices were selected as the base matrix for developing solid-phase protein PEGylation system.

4.2.1 Carboxylated solid matrices for PEG grafting

As mentioned in Section 2.6.2, two different types of matrices were used to study their influence on the properties of the solid-phase system, namely hydrophilic Sephadex derivatives such as carboxymethyl

Sephadex (CM Sephadex) and carboxypentyl Sephadex (CP Sephadex) and hydrophobic polystyrene derivative. Carboxylated polystyrene ((Succinylaminoethyl) polystyrene, Polystyrene AM COOH) was procured directly from Sigma-Aldrich whereas the Sephadex G 25 coarse (obtained from GE healthcare Ltd., Uppsala, Sweden) was carboxylated with a suitable linker for further modifications.

Carboxylated Sephadex derivatives prepared for grafting studies were characterized by the difference in their carboxyl tether lengths. In one modification, CM Sephadex was prepared with a single carbon carboxy tether, whereas in another modification CP Sephadex was prepared with a five-carbon tether. These differences in the carbon chain length were used as a measure to study the influence of tether length on the PEG grafting as well as to study the steric repulsive interactions due to crowding at the reaction site during grafting.

4.2.1.1 CM Sephadex

Carboxymethylation of Sephadex using monochloroacetic acid has been extensively reported (Flodin 1962; Fujita et al. 1981; Mauzac et al. 1982; Carreno et al. 1988) due to applications in gel filtration and ion exchange chromatography. All this literature followed methodologies similar to that initially reported by Flodin (1962) using benzene as the reaction media. The very high carcinogenicity associated with benzene and the heterogeneous reaction mixture which occurs when it is mixed with aqueous alkaline media necessitates the requirement of a safer and a water soluble protic solvent for Sephadex modifications.

The carboxymethylation of Sephadex structural analogues such as dextran (Hattori et al. 1994; Nogusa et al. 1994; Tsujihara et al. 1997;

Huynh et al. 1998), chitin (Wan et al. 1996; Hjerde et al. 1997; Sakairi et al. 1998) and chitosan (Liu et al. 2001; Chen and Park 2003; Liang and Zhang 2007) was extensively studied and the influence of solvent composition on the reaction as well as product profile was also well documented (Chen and Park 2003). A method for the modification of Sephadex which incorporated the optimised reaction parameters reported for the carboxymethylation of chitosan by Chen and Park resulted in a better carboxyl content and product profile than those following Flodin's method and was thus adopted throughout the project.

4.2.1.2 CP Sephadex

In search of a relatively longer tether to study its influence on the PEG grafting profile, a carboxypentyl derivative having a five carbon spacer was chosen for modification. Experiments were performed by following the same optimized process for the preparation of CM Sephadex using 6-bromohexanoic acid in place of monochloro acetic acid.

4.2.2 PEG grafting

PEGs can be grafted onto various surfaces using a variety of methods which were reviewed in Section 2.7. In view of the requirement for a cleavable linkage between grafted PEG and the solid matrix, covalent grafting of PEG onto the matrix was chosen. Covalent grafting of PEG onto solid surfaces can be performed under either aqueous conditions or non-aqueous conditions.

4.2.2.1 Aqueous PEG grafting

Covalent coupling of PEG derivatives such as amines, aldehydes and carboxylic acids can be successfully performed with various functionalized solid matrices under aqueous conditions and this is one of the most frequently used grafting methods for preparing PEG modified surfaces. Commonly reported aqueous-phase coupling methods for PEG amines include grafting through amide bonding onto carboxylated surfaces such as polystyrene (Zalipsky et al. 1994; Van Delden et al. 1996; Meng et al. 2004a; Meng et al. 2004b), silicon wafers (Andruzzi et al. 2007), nanoparticles (Popielarski et al. 2005; Cheng et al. 2007; Pan and Feng 2008) and gold (Feng et al. 2005), and sodium cyanoborohydride mediated coupling with aldehyde functionalized surfaces like glass (Schlapak et al. 2006). In a similar way, carboxy functionalized PEGs can be attached to amine functionalized surfaces following the carbodiimide chemistry (Wazawa et al. 2006; Hong et al. 2007; Thierry et al. 2008; Barrera et al. 2009), as well as a PEG aldehyde derivative onto an amine modified surface catalysed by sodium cyanoborohydride (Saneinejad and Shoichet 1998; Kingshott et al. 2002; Kingshott et al. 2002; Cole et al. 2007).

Covalent coupling of PEG amine with a carboxyl group can be performed in two different ways. In one method the carboxyl terminal of the surface can be preactivated using a suitable functional group, followed by reacting with the amine functionalized PEG, whereas in the second method, which is a single-pot method, the activation and coupling will be carried out simultaneously in a single solution. These two methods are general methods for amine coupling and are applicable irrespective of whether the reactions are being carried out in aqueous or non-aqueous conditions. In aqueous-phase reactions, normally the two step coupling

process involves the carboxyl activation being catalysed by a water soluble carbodiimide, usually 1-ethyl-3-(3-dimethylamino) propyl carbodiimide (EDC), followed by coupling with the amino PEG (Van Delden et al. 1996; Meng et al. 2004a; Meng et al. 2004b; Andruzzi et al. 2007). In the case of the single-pot coupling method, the amine derivative, EDC and the activating agent are mixed together with the carboxylated surface in a suitable buffer to achieve the coupling (Popielarski et al. 2005). The most specific and commonly used activating agents for amine coupling are N-hydroxysuccinimide (NHS), N-hydroxysulfosuccinimide (sulfo-NHS) and N-hydroxybenzotriazole (HOBt) (Hermanson 1996).

Van Delden et al (1996) reported almost double the surface concentration of PEG on carboxylated polystyrene beads when it was grafted through the single-pot coupling method compared with following the two step pre-activation method using a 1000 Da PEG amine (Figure 4-1). However, the application of this single-pot method for grafting the heterobifunctional PEG derivative, in this present context with an amine terminal at one end and a carboxyl terminal at the other end, can create complexity and non-specificity if both catalyst and activating agents are present along with this PEG derivative. Hence pre-activation of surface carboxyl groups, followed by reaction with PEG amine was selected for aqueous-phase PEG grafting.

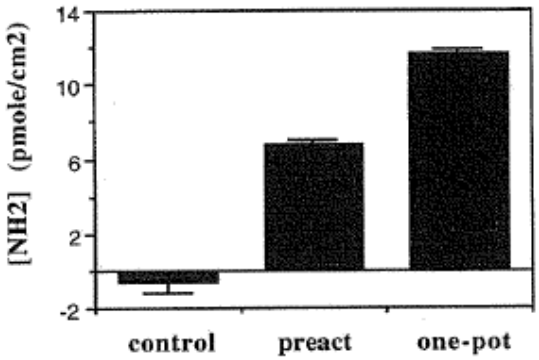


Figure 4-1: PEG surface concentration as measured as concentration of amino groups obtained from diamino-PEG derivative (1000 Da)(Van Delden et al. 1996).

A detailed study of PEG amine grafting onto carboxylated polystyrene matrices was conducted by Meng et al. (2004a; 2004b) and Van Delden et al (1996). They suggested that grafting kinetics were greatly influenced by a number of parameters such as the pH of the reaction medium, coupling time and the relative molar equivalents of the carbodiimide catalyst and surface COOH concentrations. The influence of pH on carboxyl activation and PEG coupling is illustrated in Figure 4-2, in which zeta (ζ) potential correlates to the surface PEG concentration, while an increase in concentration leads to a less negative ζ-potential. The results suggest that a slightly acidic pH between 5.5 and 6 favours the carboxyl activation (pK_a of surface COOH groups) whereas a slight alkaline pH between 8.5 and 9.5 (close to the pK_a of PEG amine) favour the PEG coupling.

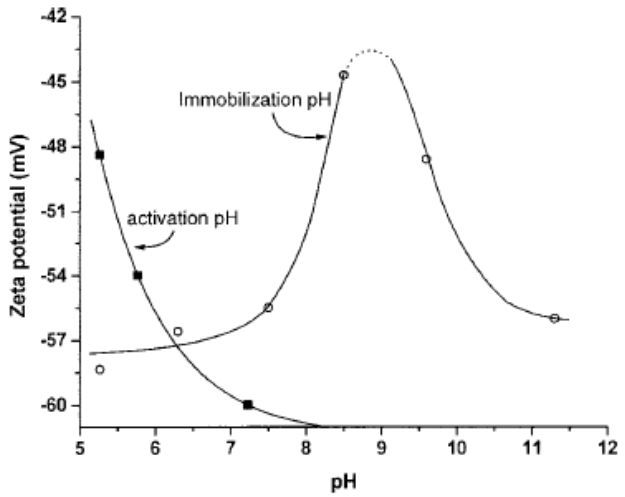


Figure 4-2: Influence of pH on activation and PEG amine coupling (Meng et al. 2004a)

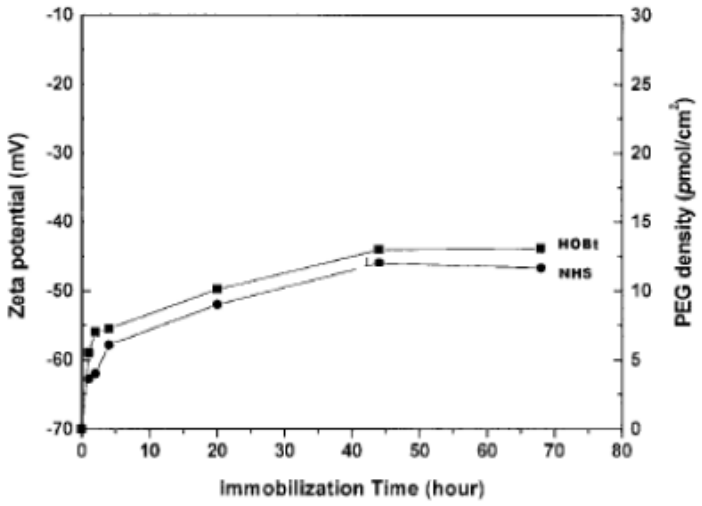


Figure 4-3: Influence of coupling time on PEG concentration (Meng et al. 2004a)

Figure 4-3 illustrates the influence of coupling time on PEG grafting, and shows an initial lower concentration which increases with reaction time. After a period of time, stagnancy in reaction was observed with no further increase in PEG surface concentration even after prolonged

immobilization. This indicates a saturation condition due to increased steric repulsions between grafted and approaching PEG molecules, thereby preventing further conjugations. PEG surface concentrations also depend on the molar equivalent of the catalyst used and were found to increase with increasing catalyst concentrations. The optimization of molar equivalents by Meng et al suggests that the molar ratio of carbodiimide-COOH should be around 5 to 6 to get an optimal maximum PEG coupling on the surface.

In addition to the above mentioned parameters which were specific to amine coupling, the following important factors may also influence the grafting profiles which are related to the inherent properties of PEG chains. PEG graftings are greatly influenced by the interchain repulsive interactions between grafting and grafted PEG molecules. Studies showed that the presence of higher salt concentrations and elevated temperatures enhances higher grating densities due to reduced interchain repulsive forces resulting from the disruption of hydrated shells surrounding the PEG chains (Emoto et al. 1996; Kingshott et al. 2002; Kingshott et al. 2002). Another important factor which influences the PEG grafting profile, irrespective of the reaction media, is its molecular weight, and grafting will become increasingly difficult as the molecular weight increases. As shown in the data presented by Lu and Felix (1994) (Figure 4-4), an almost quantitative coupling yield was achieved within 5 hour with a low molecular weight PEG (750 Da), while only about 50% yield was achieved with a high molecular weight PEGs (5000 and 10000 Da) even after continuing the reaction for 72 hours.

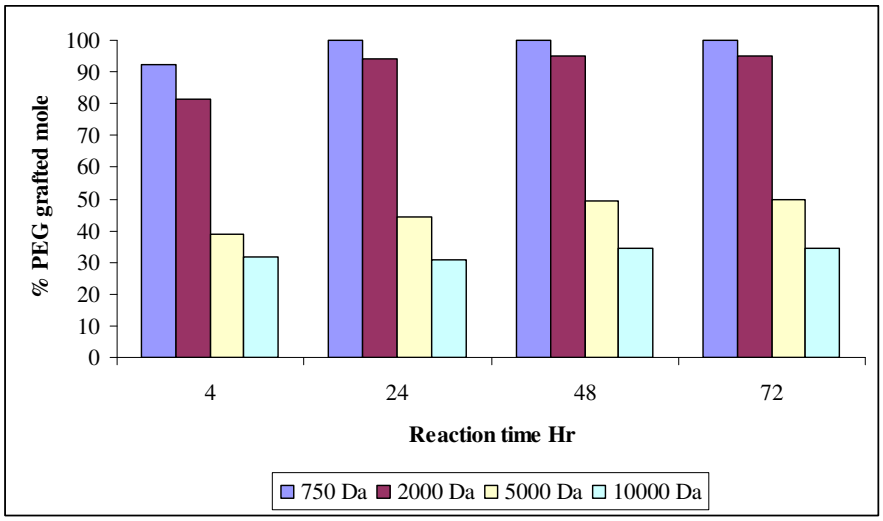


Figure 4-4: Influence of PEG molecular weight on coupling (Lu and Felix 1994)

Summarizing the optimized parameters for aqueous-phase PEG grafting, a prolonged coupling time and alkaline conditions are required to get a reasonable grafting profile. But the labile nature of the β-alanine ester linkage associated with the PEG derivative will result in a parallel hydrolysis of PEG, both grafted as well as reacting, and cause a net loss of surface PEG concentration. Hence the use of aqueous PEG coupling may not be a viable grafting methodology due to the specific nature of PEG in use.

4.2.2.2 Non-aqueous PEG grafting

Compared to aqueous-phase grafting, non-aqueous covalent grafting of PEG onto various matrices has not often been cited but it can also be performed either in a single-pot method or by following the preactivation methodology as in the previous case. In the single-pot methodology PEG amine was treated with the carboxy functionalized surface in the presence of a carbodiimide (Ploehn and Goodwin 1990; Chattopadhyay et al. 2006), whereas in the latter method carboxyl groups were preactivated

with a suitable activating agent in the presence of a catalyst followed by reacting with the PEG amine derivative in the presence of a base (Riener et al. 2003; Halpin and Harbury 2004).

Due to the specificity and selectivity of the preactivated method for amine coupling in the presence of other functional groups, this methodology was preferred over the single-pot one and followed for grafting β-alanine PEG derivatives in this project. This non-aqueous preactivation method was used as a useful conjugation methodology for PEG amine derivatives in solution-phase coupling reactions and was followed for the preparation of many drug delivery applications (Cho et al. 2005; Kim et al. 2005; Paranjpe et al. 2005).

4.2.3 Hydrolysis of the grafted PEG

The hydrolysis profile of a β-alanine ester linkage under homogenous solution conditions was described in Section 3.2.1.3. Even though we can anticipate a reasonable difference in hydrolysis nature for a heterogeneous surface bound alanine ester, a quite similar hydrolysis yield of about 85% was reported for the hydrolysis of an alanine ester linkage attached to a Sepharose derivative using 0.3 M NaOH solution after 15 minutes (Wilchek and Miron 1987). The compilation of these results regarding the labile nature of β-alanine ester linkages provided a very promising synthetic strategy and become the major backbone of the proposed solid-phase PEGylation approach.

In the absence of any UV absorbance by the PEG molecule alone, a UV active chromophore modified PEG derivative was a better choice for the quantitative estimation of the hydrolysed PEG from the matrix (Section

3.2.2). Based on these, an α-(β-alanine)-ω-benzyl amide PEG derivative (**14**) was prepared (Section 3.5.2) for hydrolysis kinetic studies.

4.2.4 Solid-phase protein PEGylation

As illustrated Figure 2-19, the solid-phase PEGylation system consists of a covalently grafted PEG derivative onto a functionalized solid matrix through a cleavable linker, with the free end terminal available for protein conjugation. Activation of the carboxyl PEG terminal with NHS ester was preferred for further protein conjugation, as followed in the case of solution-phase PEGylation (Section 3.2.3). Protein conjugated PEG derivatives were then cleaved off from the matrix under mild conditions and were characterized using SEC.

4.3 Materials

4.3.1 Reagents and solvents

All PEG derivatives were prepared following relevant methodologies described in Chapter 3. In addition to those chemicals and solvents mentioned in Section 3.3 (Table 3-3 and 3-4); ethanolamine, glycine, hydroxylamine hydrochloride, monochloroacetic acid (MCA), 1-ethyl-3-(3-dimethylamino)propyl carbodiimide hydrochloride (EDC.HCl) (all were purchased from Sigma-Aldrich), isopropanol (IPA), sodium hydroxide (NaOH) (from Merck, Darmstadt, Germany), 6-bromohexanoic acid (from Fluka, Buchs, Switzerland) and sodium chloride (NaCl) (from Ajax Finechem Ltd.) were used in surface modifications and PEG graftings. Sephadex G 25 coarse was purchased from GE healthcare Ltd., and polystyrene AMCOOH was from Sigma-Aldrich.

4.3.2 Buffers

10mM PBS was prepared by following the method given in Section 3.3. Other buffers were prepared by using methods given below.

4.3.2.1 Glycine-NaOH

Stock solutions of 0.2 M glycine and 0.2 M NaOH were prepared in Millipore water. The pH of the glycine solution was adjusted to pH 8.5 with dropwise addition of NaOH stock solution under stirring. It was then stored at 4°C.

4.3.2.2 Hydroxylamine-NaOH

Stock solutions of 0.2 M hydroxylamine hydrochloride and 0.2 M NaOH were prepared and pH of the hydroxylamine solution was adjusted with NaOH solution to 8.5 and immediately used without any further storage.

4.4 Analytical methods

4.4.1 FTIR

IR spectra using KBr disk were recorded on a Perkin-Elmer System 2000 FTIR spectrophotometer (PerkinElmer, Massachusetts, USA). Typically 64 scans per spectrum were recorded at 4 cm^{-1} resolution.

4.4.2 AKTA Explorer

A detailed description of AKTA explorer is given in Section 3.4.3. For analysis of α-hydroxy-ω-benzyl PEG derivative (**11**) and monitoring of

the hydrolysis kinetic studies 10% methanol (in DI water) was chosen as the elution medium instead of PBS buffer.

4.4.3 UV spectrophotometer

UV absorbances were measured by using an Ultraspec 2100 pro UV/Visible spectrometer (GE Healthcare Ltd., Uppsala, Sweden). Measurements were carried out at 280 nm for Cytochrome c solutions and a calibration curve was plotted with known concentrations against their absorptions. The concentration of unknown solutions was calculated by comparing their absorption with the calibration curve using the same buffer solution.

4.4.4 Optical microscope

Diameters of Sephadex and polystyrene matrices before and after PEG grafting under both dry and wet conditions were measured with an Olympus BX60 optical microscope (Olympus Optical Co., Ltd, Tokyo, Japan) using an UMPlanFI 10x / 0.30 BD lens. Diameters of a number of particles were measured in pixels which were further calibrated with a graduated optical scale under the same focal plane and length and converted into micrometres.

4.4.5 Acidimetric estimation of the carboxyl content of Sephadex derivatives

0.25 g Carboxylated Sephadex was stirred with 0.05 M sodium bicarbonate solution (25 mL) for 48 hour at room temperature. The mass was filtered and the clear filtrate was titrated against 0.05 M HCl solution using methyl red indicator to estimate the amount of sodium bicarbonate

consumed by the resin. A blank experiment was performed with unmodified Sephadex by exactly following the same conditions. From this the acid content (moles per g) of the modified matrix was calculated.

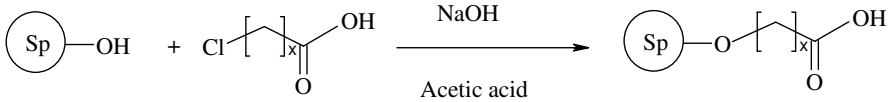
4.5 Experimental methods

The overall experimental methods for preparing the solid-phase PEGylation system can be broadly classified into the preparation of a carboxyl modified matrix (except polystyrene), its NHS activation, selective covalent coupling between the NHS activated carboxylated surface and the NH_2 terminal of alanine modified PEG, blocking of the unreacted NHS activated carboxyl groups with ethanolamine, NHS activation of the carboxy terminal of grafted PEGs, its conjugation with N-terminal lysine group present in the model protein Cytochrome c and finally cleaving of the PEGylated Cytochrome c from the matrix under mild conditions.

4.5.1 Sephadex modifications

Sephadex G 25 coarse was dried at 100°C overnight before modifications and stored in a desiccator. 10 g dried Sephadex was treated with a solution of NaOH (9 g) in 80% aqueous IPA (180 mL, 18 vol.) at 55°C for 2 hour. A solution of monochloroacetic acid (11.2 g) in 80% aqueous IPA (20 mL, 2 vol. of Sephadex) was prepared and added into the reaction mass drop wise under stirring over a period of 30 minutes, and maintained at the same temperature for further 4 hour. After cooling to room temperature the reaction mass was quenched into 70% aqueous ethanol and stirred for 30 minutes. Solids were filtered over a Buchner funnel and washed subsequently with 70% aqueous ethanol (2 x 100 mL), DI water (2 x 100 mL) and finally with ethanol (2 x 100 mL).

The above prepared sodium salt of carboxymethyl Sephadex was resuspended in 80% aqueous ethanol and neutralised slowly with the addition of acetic acid. The mass was stirred for a further one hour with occasional addition of acetic acid to maintain the pH if necessary. The product was filtered and washed thoroughly with 80% aqueous ethanol (2 x 100 mL), DI water (2 x 100 mL) and finally with ethanol (2 x 100 mL). The CM Sephadex derivatives were then freeze dried till a constant weight was achieved, and stored at 4°C.



(17)

x = 1 for CM Sephadex
5 for CP Sephadex

Figure 4-5: Scheme-6 Preparations of Sephadex carboxyl derivatives

The same synthetic scheme (Figure 4-5) was followed for the preparation of CP Sephadex derivative using 6-bromohexanoic acid (23.1 g, 0.12 moles, equivalent to 11.2 g MCA) in place of monochloroacetic acid.

4.5.2 PEG grafting

The PEG grafting of carboxylated solid matrices was performed by first converting the carboxyl terminals into their NHS esters followed by reacting with the amine terminal of alanine-PEG derivatives. Figure 4-6 illustrates the detailed synthetic scheme for PEG grafting onto the various matrices under investigation.

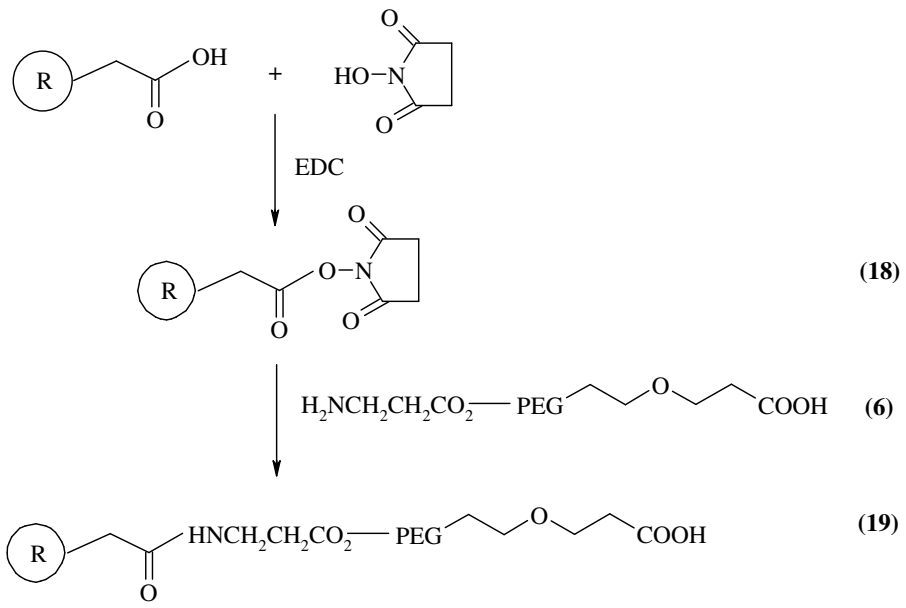


Figure 4-6: Scheme-7 Synthetic Scheme for PEG grafting

In the optimised and general method, carboxylated matrices were first converted into their corresponding NHS esters by treating them with 10 molar equivalents of NHS (based on the carboxyl content of matrix) in the presence of EDC.HCl (10 eq.) in anhydrous DMF (20 vol. of solid matrix) under an N_2 atmosphere. After stirring the reaction mixture for 4 hour at 20°C, the matrix was separated, washed thoroughly with anhydrous DMF (2 x 5 vol.) and used immediately for the subsequent step.

The optimised PEG grafting procedure involves reacting NHS activated matrix with 5 molar equivalent amount β-alanine PEG derivative (based on the carboxyl content of original matrix) in the presence of DIEA (10 eq.) in anhydrous DMF (1.5 vol. of PEG amount) under an N_2 atmosphere at 40°C for 72 hour. After the reaction, unreacted PEG derivatives were removed by washing thoroughly with anhydrous DMF (2 x 20 vol.).

4.5.3 Hydrolysis of grafted PEG

Hydrolysis kinetics was monitored by grafting α-(β-alanine)-ω-benzyl amide PEG derivative (**14**) onto the NHS activated CH Sephadex following the non-aqueous grafting method. Hydrolysis was performed using 0.2 M glycine–NaOH (pH 8.5), 0.2 M hydroxylamine–NaOH (pH 8.5), 0.2 M acetate (pH 4.5) buffers and 0.2% TFA (pH 2.35) under various conditions.

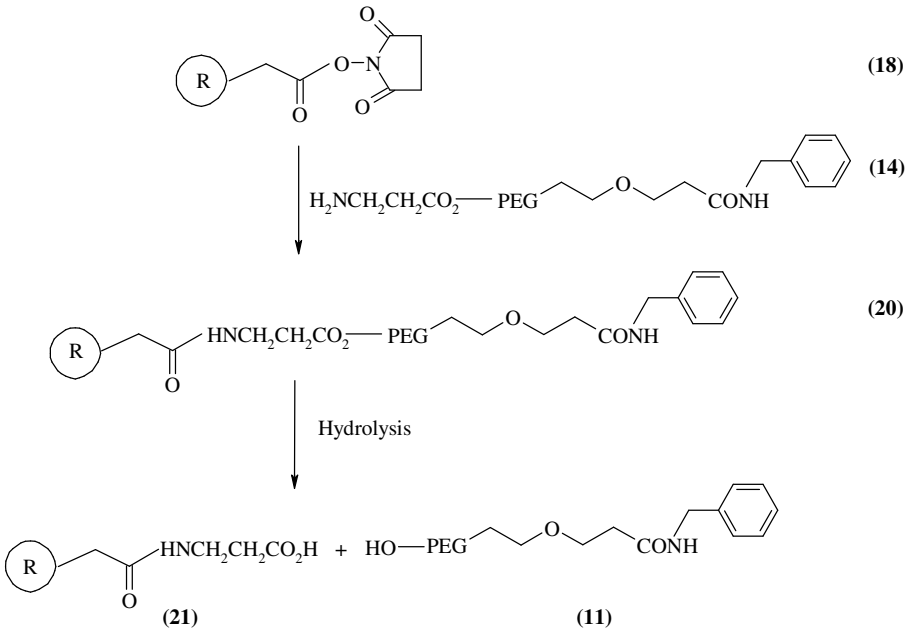
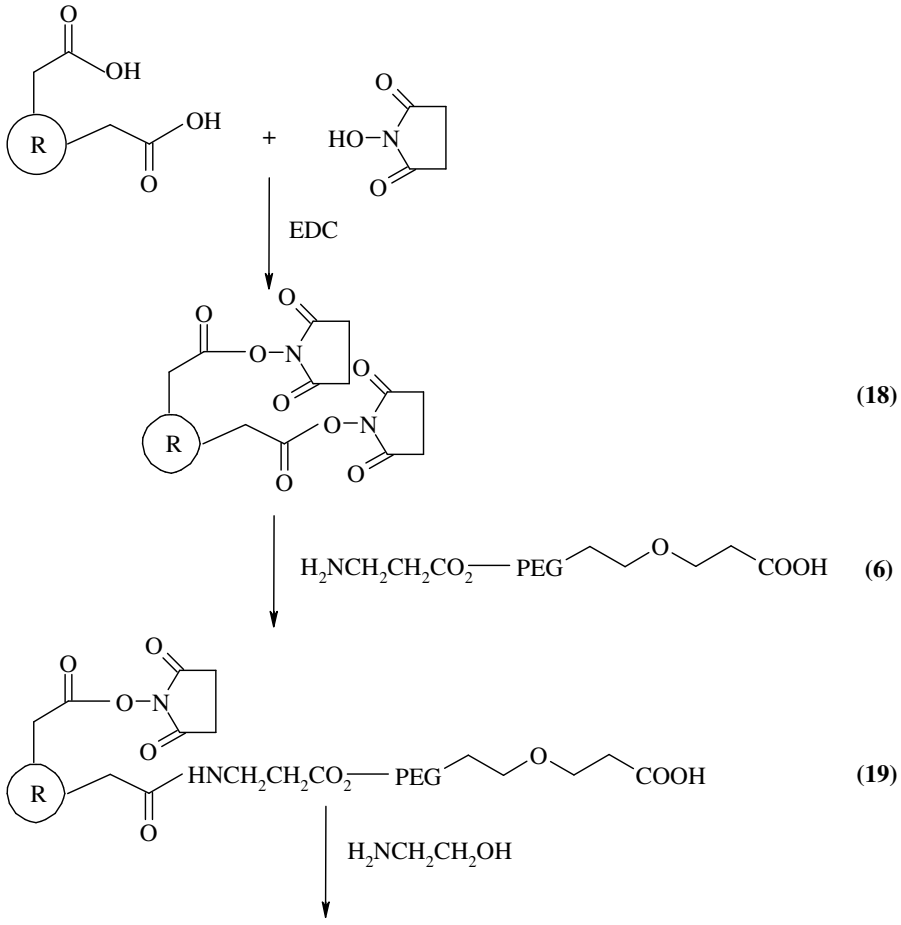


Figure 4-7: Scheme-8 PEG grafting and hydrolysis

Hydrolysed fractions were separated by centrifuging the matrix at 4°C. The matrix was then thoroughly washed with 2 M NaCl solution (2 x 20 vol.) followed by DI water (2 x 20 vol.) and all the combined fractions were concentrated by freeze drying under high vacuum. The concentrated residual mass was re-dissolved in 1 mL 10% methanol and analysed by SEC following the method given in Section 3.4.3.

4.5.4 Solid-phase protein PEGylation

After incorporating the optimised grafting and hydrolysis conditions, Scheme-7 (Figure 4-6) was further extended to afford solid-phase protein PEGylation as illustrated in Scheme-9 (Figure 4-8). NHS ester preparation of carboxylated matrices and subsequent β-alanine PEG coupling were performed following the method given in Section 4.5.2 and the corresponding PEG grafted solid matrices **(19)** were prepared.



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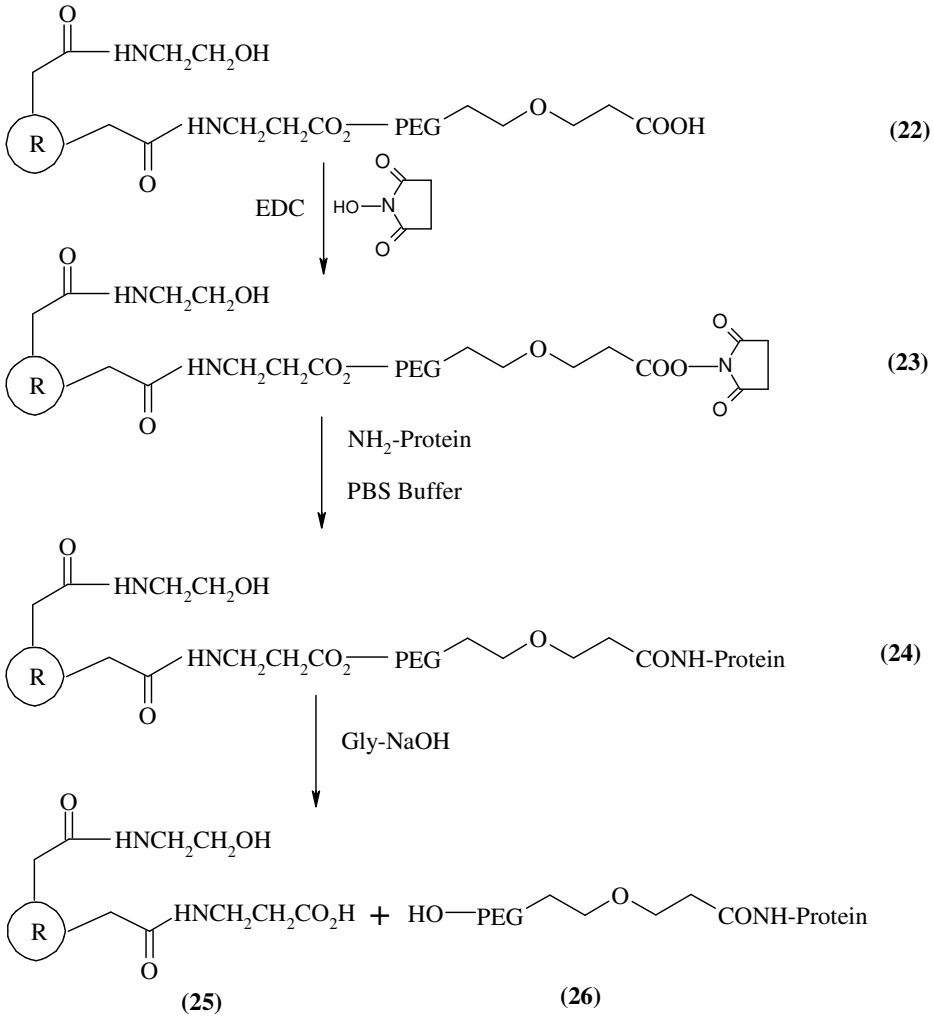


Figure 4-8: Scheme-9 Solid-phase protein PEGylation

4.5.4.1 Ethanolamine blocking

Blocking of unreacted NHS activated carboxyl groups present with the PEG grafted intermediate **19** was performed by treating with excess ethanolamine (15 eq. based on carboxyl content of the original matrix) in the presence of DIEA (30 eq.) in anhydrous DMF (20 vol. of solid matrix) under an N_2 atmosphere at 40°C for a further 12 hour. After the

reaction, the matrix was washed with DMF (2 x 20 vol.) and was further washed thoroughly by sonicating with DI water (2 x 20 vol.), 2 M NaCl solution (2 x 20 vol.) and finally again with DI water (2 x 20 vol.). The PEG grafted product was then freeze dried until a constant weight was achieved and stored under N_2 atmosphere at 4°C.

4.5.4.2 NHS activation of PEG carboxyl terminal

Carboxy functionalized PEG grafted solid matrices **(22)** were treated with 5 molar equivalent of NHS (based on the carboxyl content of the original base matrix) in the presence of EDC.HCl (5 eq.) in anhydrous DMF (20 vol. of solid matrix) under an N_2 atmosphere overnight. After this, the matrix was washed with DMF (2 x 5 vol.) and PBS buffer (2 x 5 vol.) at 4°C and used immediately for protein conjugation.

4.5.4.3 Cytochrome c conjugation

NHS activated PEG grafted matrices **(23)** were immediately reacted with a solution containing 5 mg/mL Cytochrome c in PBS buffer (1 mL for every 50 mg PEG grafted matrix) at pH 7.4 for 2 hour at 20°C. Clear filtrate was separated after the reaction by centrifuging the material at 4°C (used for estimating unreacted Cytochrome c using UV) and the matrix was washed with PBS buffer (2 x 20 vol.), 2 M NaCl (2 x 20 vol.) and finally with PBS buffer (2 x 20 vol.).

4.5.4.4 Hydrolysis and release of PEGylated Cytochrome c

Cytochrome c conjugated PEG grafted matrices **(24)** were treated with 0.2 M glycine-NaOH buffer (pH 8.5) at 20°C for 24 hour. The hydrolysed PEGylated **(26)** fractions were separated by centrifuging the

matrix at 4°C. The matrix was washed with 2 M NaCl solution (2 x 20 vol.) and with DI water (2 x 20 vol.) and all the combined fractions were freeze dried and the concentrated residual mass redissolved in 1 mL PBS buffer and analysed by SEC following the method given in Section 3.4.3.

4.6 Results and discussion

4.6.1 Carboxylated solid matrices

Carboxy derivatives of Sephadex were prepared by modifying its hydroxyl groups with carboxylic tethers having varying chain lengths. Initial experiments were done by following the procedure of Flodin (1962) using benzene as the solvent. Even though carboxymethyl derivatives with a comparable ion exchange capacity to that of the reported literature (Flodin 1962; Armarego and Chai 2003) were prepared, better results and product profile were obtained after using 80% IPA solution instead of benzene. The carboxymethyl content was estimated by using the acidimetric titration (Section 4.4.5) and the summary of results is given in Table 4-1.

Table 4-1: Carboxymethylation of Sephadex using different solvents

Solvent	**Ion exchange capacity (millieq./g)**	
	In house analysis	**Reported**
Benzene	2.95 ± 0.19	3.0 [*]
		4.5 ± 0.5 [#]
80% IPA	6.30 ± 0.36	

[*]Using benzene (Flodin 1962) and [#] Solvent unknown (Armarego and Chai 2003)

During carboxymethylation using benzene according to Flodin's method, a heterogeneous reaction mixture and the formation of lumps were

observed, owing to the immiscible nature of the solvents and the subsequent uneven distribution of the reagents. However, with aqueous IPA solution, the Sephadex beads were swelled enough to allow a uniform contact with the reagents and were distributed homogeneously throughout the reaction mass, resulting in an increased carboxyl content of the product obtained.

CP Sephadex prepared from 6-bromohexanoic acid using 80% IPA solution resulted in a carboxyl content of 0.98 ± 0.07 millieq./g. The acid content of PS AM COOH procured from Sigma-Aldrich was also estimated following the same method and was found to be 0.91 ± 0.11 milliequivalents/g (reported as per COA: 0.8 – 1.2 millieq./g).

4.6.2 NHS ester preparation

Optimization of the reaction parameters was performed with CM Sephadex and the optimised process was later extended to other matrices. As convincing evidence to quantify NHS formation, NHS activated resins were treated with a known quantity of Cytochrome c in PBS buffer (Section 4.5.4.3) and the amount of Cytochrome c conjugated onto the activated resin was estimated by comparing the UV absorptions of Cytochrome c solution before and after conjugation against a standard curve of known concentrations (Section 4.4.3).

4.6.2.1 EDC-NHS molar ratio

Initial experiments were performed with approximately equimolar amount of reagents, but later experiments were optimised with 10 molar equivalents of EDC and NHS to get maximum NHS activation of the available carboxyl groups (Figure 4-9). Reaction saturation was observed

with 10 equivalents and more, hence further experiments were performed with 10 molar equivalents of EDC and NHS.

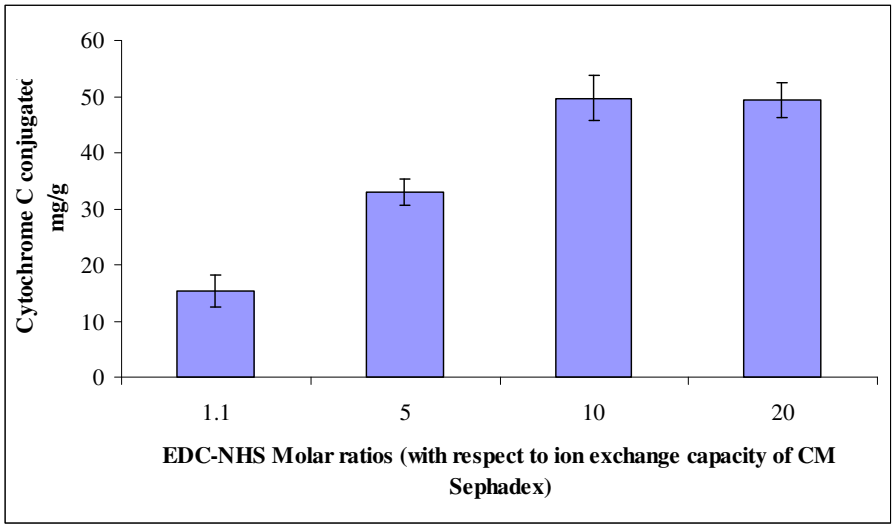


Figure 4-9: Influence of EDC-NHS molar ratio on NHS preparation

4.6.2.2 NHS ester preparation of other matrices

As with the CM Sephadex derivative, NHS esters of CP Sephadex and polystyrene were also conjugated with Cytochrome c and the results are tabulated in Table 4-2. Even after prolonged conjugation and the use of varying amount of salts, no protein conjugation was observed onto the polystyrene matrix due to its hydrophobic nature, which may retard the approaching protein for the conjugation (Wang et al. 1995).

Table 4-2: Cytochrome c conjugated to CP Sephadex and polystyrene matrices (10 eq. EDC/NHS)

Resin	Amount of Cytochrome c conjugated onto the resin mg/g
CP Sephadex	14.46 ± 0.64
Polystyrene COOH	Nil

4.6.3 PEG grafting

Grafting of the alanine PEG derivative onto carboxylated matrices was studied in detail to obtain the maximum grafting density and the preferred PEG brush conformation in view of the proposed solid-phase protein PEGylation. Literature cited the requirement of a very high molar excess of PEG derivatives ranging from 2 to 150 equivalents (Zalipsky et al. 1994; De Cuyper et al. 2002; Meng et al. 2004a; Meng et al. 2004b; Popielarski et al. 2005; Pan and Feng 2008) to obtain a satisfactory grafting density on the surface, depending on the nature of the matrix. To have uniformity in the process and also due to the operational constraints, five molar equivalents of PEG-alanine derivative was used for surface grafting with all matrices.

FTIR spectra were recorded initially to confirm the presence of PEG on the solid matrix. IR spectra of polystyrene and PEG grafted polystyrene (PEG 4000 Da, 72 hr, 25°C) for example are given in Figure 4-10. The characteristic broad peak around 2900 $cm^{-1}$of PEG back bone arising from the strong absorption of $–CH_2-CH_2-$ stretching, and also the broad peak occurring at 1000 to 1100 cm^{-1} due to the characteristic -C-O-C- stretching of PEG moiety, confirms the presence of PEG molecules on the surface (Alcantar et al. 2000; Xu et al. 2003; Feng et al. 2005; Mert et al. 2008).

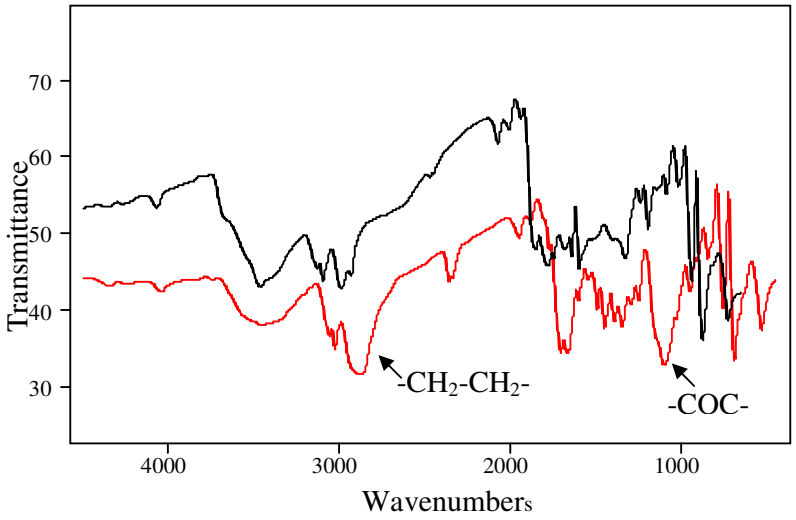


Figure 4-10: IR spectra of polystyrene and PEG grafted polystyrene (——— PS and ——— PSPEG)

Due to the lack of quantification capability with the available FTIR instrument and also due to the detailed surface characterization using the highly sensitive XPS technique (Chapter 5), no efforts to estimate the amount of grafted PEG using FTIR were made. A relative quantification method was adopted in which the gain in weight of the matrix after modification was directly correlated to the amount of PEG grafted onto the surface. In order to have maximum accuracy and minimum variations between the samples, experiments were repeated at least three times and the resins were extensively washed to remove any adhered PEG molecules on the surface. Blank experiments were performed with the corresponding NHS esters and were worked up following the same procedure. Samples were freeze dried till a constant weight was achieved and the average of all these results was taken as the quantitative measure of the amount of PEG grafted. But later these results were validated using XPS and a close linearity was found between both the results.

4.6.3.1 Influence of base and solvent

Among the various available polar aprotic solvents, those with higher dielectric constants such as acetonitrile (36.6), dimethyl formamide (38.3) and dimethyl sulfoxide (47.2) were initially chosen for PEG grafting. DIEA, TEA and pyridine were selected as base to catalyse the PEG conjugation and graftings were performed at 25°C for 72 hours under an N_2 atmosphere. Even though the overall difference is relatively very small (Figure 4-11), combination of DMSO with DIEA resulted in maximum PEG grafting followed by DMF-DIEA. But the recovery of PEG derivative was difficult from its DMSO solution, as the recovered product became amorphous and of a waxy nature, which requires repeated crystallization for further use. But from the DMF-DIEA system, PEG derivative was recovered almost quantitatively. Consequently DMF-DIEA was selected for all PEG surface grafting experiments.

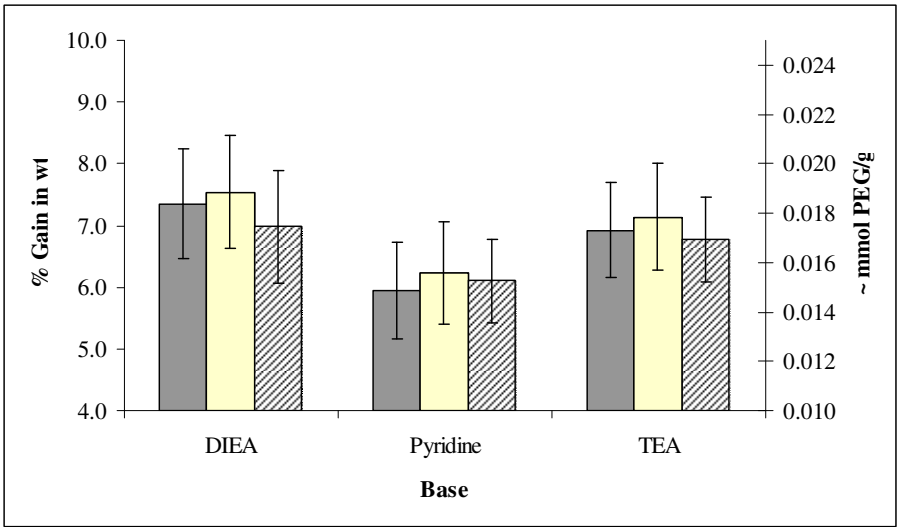


Figure 4-11: Influence of base and solvent on PEG grafting (**DMF,** DMSO and Acetonitrile)

4.6.3.2 Influence of reaction parameters and nature of matrix on PEG grafting

Grafting kinetics were found to be greatly influenced by a number of parameters such as temperature, time, molecular weight of PEG used and also by the nature of the matrix used for grafting. The impact of temperature on PEG grafting profile is illustrated in Figure 4-12, showing a substantial increase up to 40°C. But a further increase in temperature does not favour any significant improvement in grafting as it inversely affected the grafting by favouring an increased NHS ester as well as β-alanine ester hydrolysis.

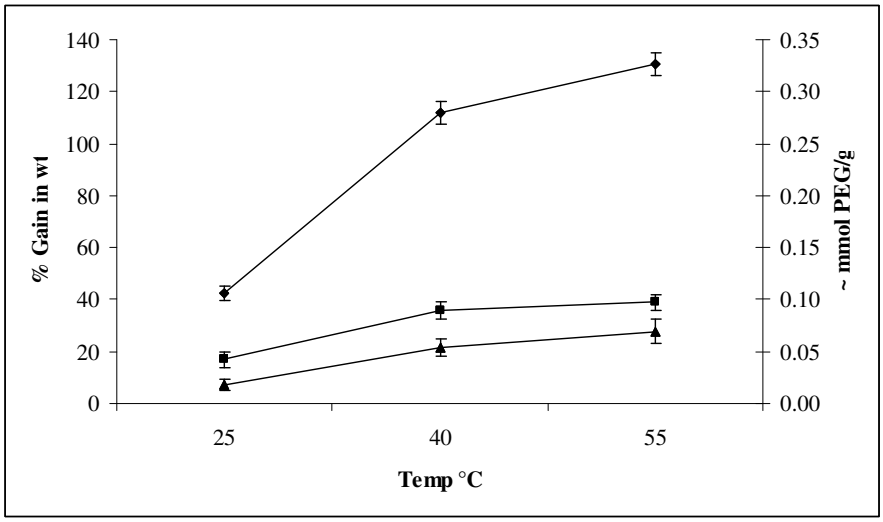


Figure 4-12: Influence of temperature on grafting profile for PEG4000 after 72 hr in terms of gain in wt. (—◆—PS4000, —■— CP4000 and —▲—CM4000)

With all resins, PEG grafting increases with time at a given temperature and it reaches saturation after a certain period of time (Figure 4-13 and Figure 4-14). As the reaction proceeds there is an increase in steric hindrance and repulsive forces exerted by the already grafted PEG molecules on the approaching molecules, and hence further conjugation

becomes virtually impossible after a period of time. These steric repulsive forces are more noticeable with CM Sephadex compared with CP Sephadex, due to the short carbon length. The grafting profiles of all these matrices (Figure 4-12 to 4-14) were found to have a similar pattern obtained from that of XPS results (Figure 5-8 to 5-10) and a detailed explanation for these patterns is given in Section 5.4.1.3.

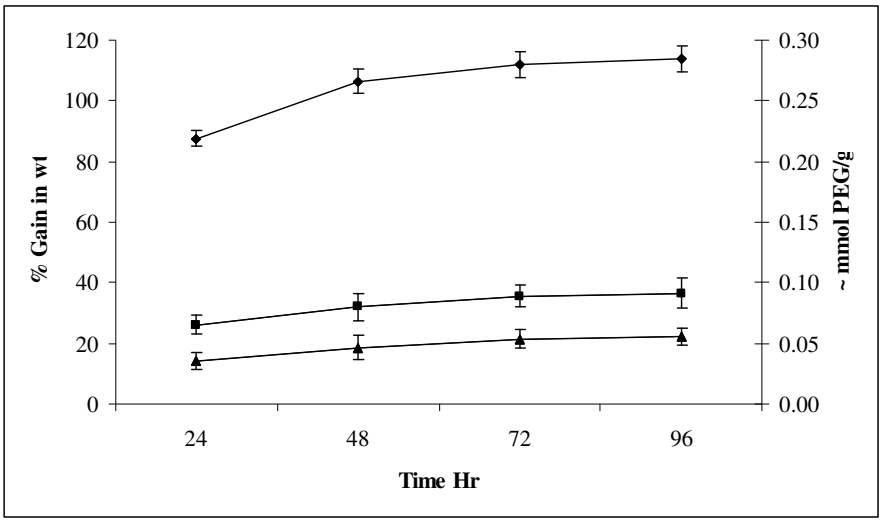


Figure 4-13: Grafting profile for PEG4000 at 40°C in terms of gain in wt. (—◆—PS4000, —■— CP4000 and —▲—CM4000)

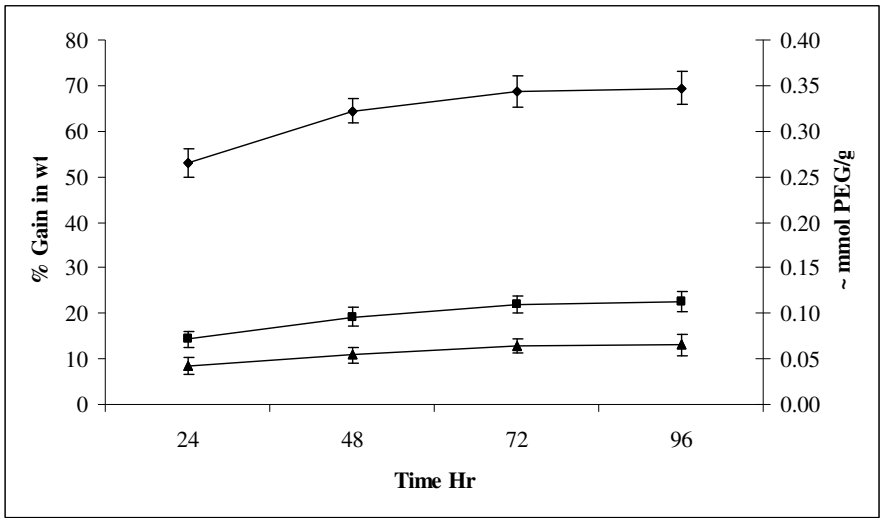


Figure 4-14: Grafting profile for PEG2000 at 40°C in terms of gain in wt. (—◆—PS2000, —■— CP2000 and —▲—CM2000)

Based on these results, covalent grafting at 40°C for 72 hour was taken as the optimised condition for preparing PEG grafted solid matrices for solid-phase protein PEGylation.

4.6.3.3 Size comparison of PEG grafted matrices and their swelling behaviour

Size comparisons of various PEG grafted matrices with their parent matrices were done under both dry and wet conditions, by measuring the diameter of a number of individual particles using an optical microscope (Section 4.4.4). Using a 10x resolution optical lens, distances in pixel were calibrated against a 10 mm graduated optical slide at a fixed focal length, and sample diameters were measured in the same focal length without disturbing the setup. The diameters of 50 single beads were measured with all dry samples and 30 beads were measured with the wet samples. The averages of all respective diameters were measured along with their standard deviation and are summarized in Figure 4-15.

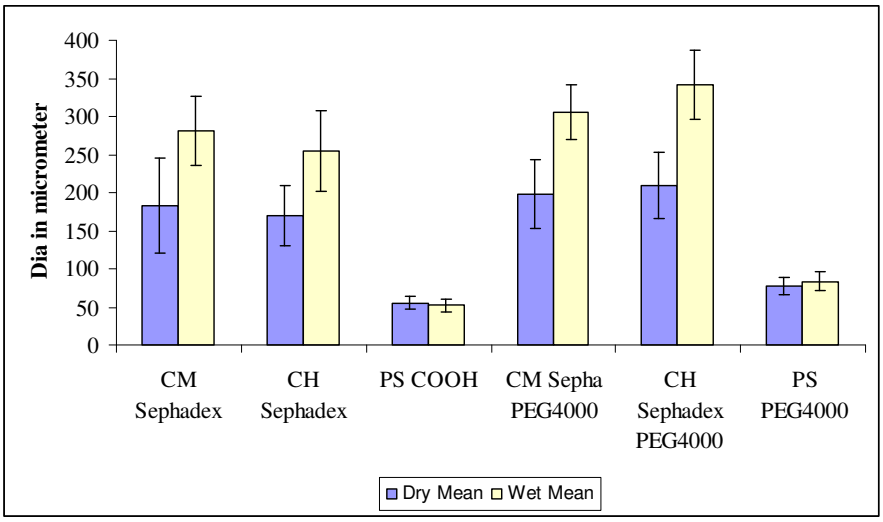


Figure 4-15: Size comparison of various PEG grafted matrices under dry and aqueous conditions (Using PEG 4000 Da, 72 hr, 40°C)

Sephadex derivatives, including those grafted with PEG, showed a very high swelling behaviour and they swelled to about 1.5 times their initial size, but the polystyrene particles remained in their initial size under aqueous conditions. On the other hand, the behaviour of these matrices was exactly reversed in non-aqueous solvents, as during experimentation it was observed that polystyrene beads swelled to almost double their initial size in anhydrous reaction media, whereas the Sephadex derivatives remained unswelled in their initial sizes. These differences in the swelling behaviour of these matrices played a key influential role in grafting kinetics as well as during protein conjugation with the solid-phase PEGylation system. The swelling behaviour of the polystyrene particles in the anhydrous reaction media resulted in maximum available sites for PEG conjugation, whereas the unswelled Sephadex matrices were left with fewer active sites for modifications. During protein conjugation, swelling of the Sephadex matrix resulted in the exposure of a number of active sites as well as unmodified carboxyl terminals, and

resulted in protein conjugation through amine coupling as well as through ion exchange interactions.

4.6.4 PEG hydrolysis

The labile nature of alanine ester linkages was well documented (Section 3.2.1.3), but the extent of its hydrolysis in heterogeneous reaction media was not exactly known. Hence it was necessary to carry out a detailed study to understand the kinetic behaviour of this linkage under various conditions, in order to assess the yield and viability of the novel solid-phase system. In order to prevent the possible protein denaturation, the main focus of hydrolysis studies was to get the maximum possible PEG hydrolysis under mild conditions such as using moderate temperature and pH.

An estimation of hydrolyzed PEG with methods such as Nessler's reagent, ammonium ferrothiocyanate and barium chloride–iodine resulted in non linear and non reproducible results, due to interferences from the presence of various reaction by-products and cleaving buffer with the hydrolyzed product. Furthermore the absence of any UV absorption by the PEG molecule alone restricts any chromatographic analysis, which normally follows UV absorption of the sample under elution for qualitative and quantitative purposes. These analytical constraints necessitate finding an alternative way of estimating the hydrolyzed PEG by modifying it with a benzyl amide chromophore, and its quantification using SEC (Section 3.2.2). A standard curve of known concentrations was plotted against peak areas of PEG benzamide derivative **(11)** obtained from SEC analysis. A standard SEC chromatogram of intermediate **11** is given in Figure 4-16. Intermediate **11** was selected for these estimations because it is the final product obtained after alanine

ester hydrolysis from the solid matrix. The peak areas of the hydrolyzed samples were compared with the standard curve and their corresponding concentrations were calculated.

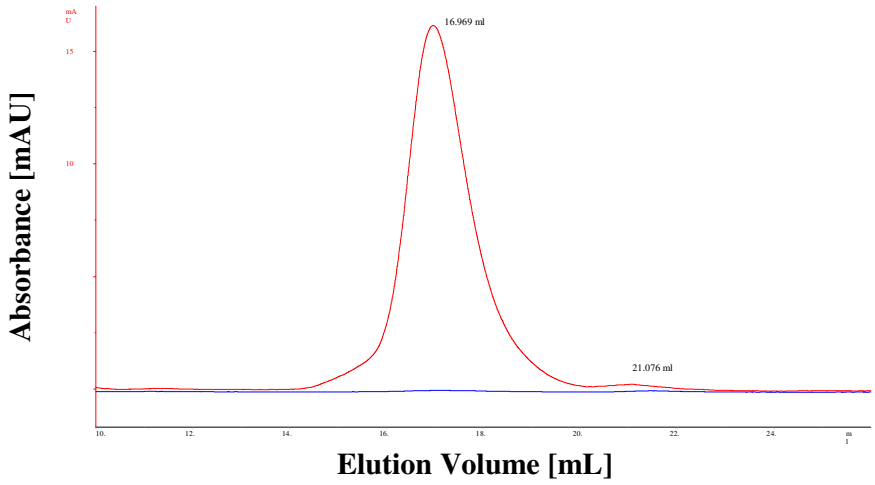


Figure 4-16: SEC of HO-PEG-CONHBz (Retention vol. 16.969 mL)

The hydrolysis profile obtained using various buffer solutions at 20°C is summarized in Figure 4-17. All other buffers except TFA resulted in a very poor hydrolysis yield even after a prolonged time. The initial rate of hydrolysis with hydroxylamine buffer was comparatively higher than that with glycine-NaOH and acetate buffer, but after a period of time there seems to be a decrease in hydrolysed PEG concentration, as shown from the graph. This is probably due to the slow reaction of hydroxylamine with amides under neutral as well as alkaline conditions (Jencks and Gilchrist 1964), which cleaves off the benzyl amide moiety from the PEG terminal and thereby results in a reduced UV absorption for the products obtained. Consequently hydroxylamine can be an alternate option for a shorter hydrolysis period, but better yields were obtained with glycine-NaOH buffer for prolonged durations.

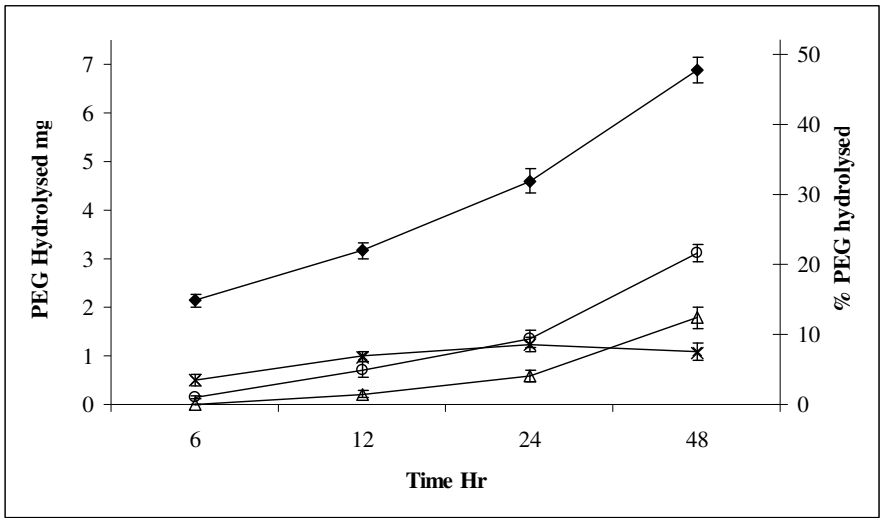


Figure 4-17: Hydrolysis using various buffers at 20°C —◆—TFA, —○—Glycine-NaOH, —△— Acetate and —×— Hydroxylamine-NaOH.

4.6.5 Solid-phase protein PEGylation

Conceptualization of the solid-phase protein PEGylation was achieved by following Scheme-9 (Figure 4-8). After PEG grafting, unreacted NHS activated resins were blocked with excess ethanolamine and further conjugated to the N-terminal lysine of Cytochrome c by NHS activation of the terminal PEG carboxyl group (Section 4.5.4). PEGylated Cytochrome c was then cleaved off from the resin by the alkaline hydrolysis of the alanine ester linkage using 0.2 M glycine-NaOH buffer at pH 8.5 and was further characterized using size exclusion chromatography.

In brief, even though there is a lack of consistency and reproducibility of results with all batches, both Sephadex based derivatives resulted in some positive results, as characterized from their SEC analysis. Typical SEC chromatographs obtained from the hydrolysis of CP Sephadex and CM Sephadex derivatives modified with PEG 4000 Da are given in Figure 4-

18 and 4-19 respectively. But none of the polystyrene based derivatives resulted in any positive results in any of the experiments. Both Sephadex derivatives were characterised by a reddish-brown coloration before and after hydrolysis, indicating a non-specific conjugation of Cytochrome c on the matrix surface, along with that through the PEG terminal. CM Sephadex derivatives resulted in very intense coloration, but the intensity was much less with CP Sephadex and was absent with polystyrene derivatives in all cases.

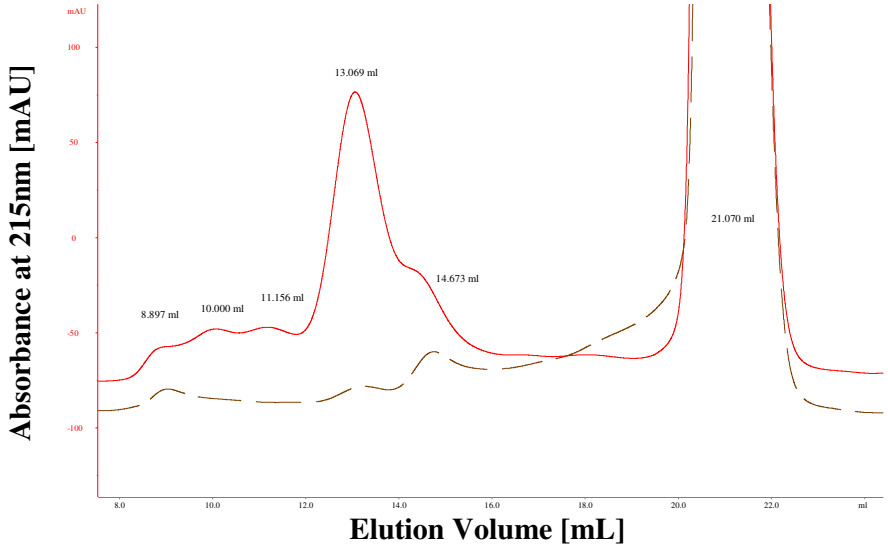


Figure 4-18: SEC of PEGylated Cytochrome c from CP Sephadex (———trial 1 and — —trial 2)

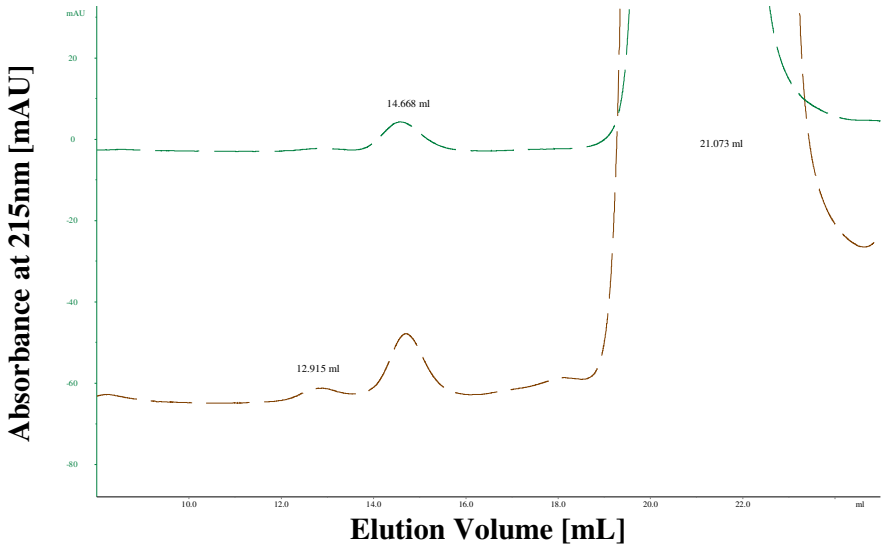


Figure 4-19: SEC of PEGylated Cytochrome c from CM Sephadex (— —trial 1 and — —trial 2)

Based on the SE chromatograph obtained from the corresponding solution-phase PEGylation (Figure 3-14), the peak for the mono-PEGylated Cytochrome c was expected to be at a column volume of ~15.8 mL. But none of these results showed any traces of mono-PEGylated species with either of the Sephadex derivatives. Peaks corresponding to column volumes ~14.6, 13, 11.2, 10 mL were detected, indicating the presence of di, tri, and multi-PEGylated species.

Size exclusion chromatograms obtained from PEG 2000 Da grafted Sephadex derivatives are given in Figure 4-20, which also shows the presence of multi-PEGylated species in their products. In order to distinguish between the various PEGylated species, fractions were pooled, freeze dried and analysed for SDS PAGE. However, due to the very low concentrations of the samples involved (as measured at 215 nm), gel pictures were not clear enough to distinguish between the various PEGylated species, and hence it was not possible to identify the products eluted at 16.17 and 14.29 mL with CP Sephadex and 15.37 mL

with CM Sephadex. Along with the PEGylated product, some Cytochrome c was also found eluted at 18.33 mL column volume with CM Sephadex. This trace amount of Cytochrome c was found with both Sephadex derivatives, in some cases even in the absence of PEGylated products, which may be due to some protein bound to the unblocked carboxyl terminal of the resin through ion exchange interactions, which may be left behind even after repeated washings.

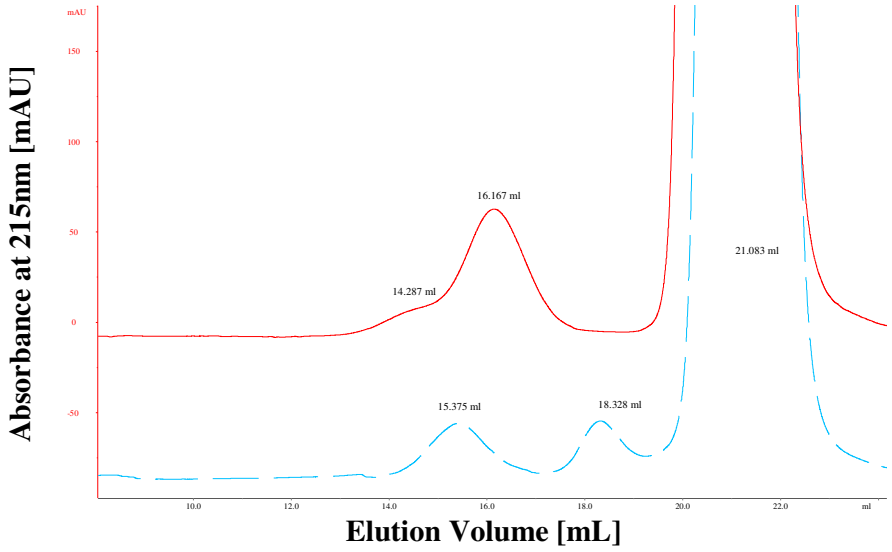


Figure 4-20: SEC of PEGylated Cytochrome c using PEG 2000 (——CP Sephadex derivative and — —CM Sephadex derivative)

SE chromatograms obtained with polystyrene derivatised with both 2000 and 4000 Da PEG derivatives does not shown any traces of the PEGylated species (Figure 4-21). They were characterized with the presence of only hydrolysed NHS by-products under all hydrolysis conditions.

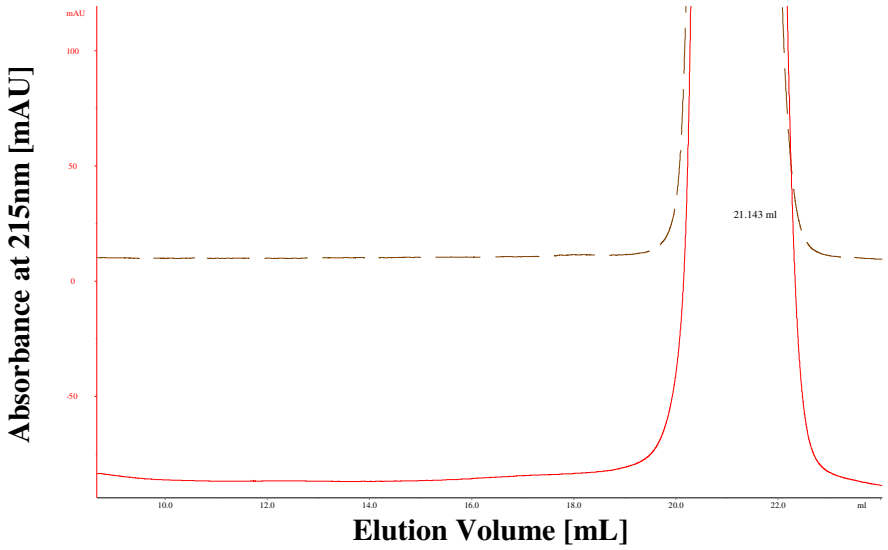


Figure 4-21: SEC obtained using polystyrene derivatives (———trial 1 and — —trial 2)

4.7 Conclusions

Carboxylated Sephadex derivatives were prepared with sufficient ion exchange capacity and tether lengths for surface PEG graftings. Non-aqueous PEG grafting methods were optimised to have a range of PEG grafted matrices with varying grafting densities, using specific amine coupling chemistry. Hydrolysis parameters were optimised to have a reasonable PEG cleaving yield under mild hydrolysis conditions in order to prevent protein denaturation.

Optimised grafting and hydrolysis conditions were extended for solid-phase protein PEGylation and the presence of traces of PEGylated species obtained from both Sephadex derivatives was characterized by SEC. Even though this provided a proof of concept for this novel technology, the process was unable to produce the expected mono-PEGylated species and is associated with lack of consistency and reproducibility in producing other multi-PEGylated products with

reasonable yield and quality. The nature of the matrix is found to have a great influence on protein conjugation and solid-phase PEGylation, as only hydrophilic Sephadex derivatives resulted positively, whereas polystyrene derivatives did not provide any positive results. Consequently, these results prompted an investigation into various factors which influenced the overall process performance and the findings are summarized in the following chapters.

5 Surface characterization of solid-phase PEGylation matrices using microanalytical tools

5.1 Introduction

In this chapter, studies on the surface characterization and morphology of PEG grafted solid matrices are described with a special emphasis on the conformational behaviour of tethered PEG molecules. Conformational behaviour of tethered PEGs can be entirely different from that of the original unbound PEG molecules and can vary with its molecular weight, grafting density and nature of the surface grafted. Because the tethering property of grafted PEG molecules is a function of their conformational behaviour, a clear understanding of conformation is necessary to study and interpret the behaviour of the newly developed solid-phase PEGylation system described in Chapter 4.

de Gennes's theory (de Gennes 1980; de Gennes 1987) postulates the correlation of grafting density of PEG chains with its preferred conformations but no systematic investigation to confirm this correlation has been reported and hence the conformation of the grafted PEG layers has merely been assumed in many cases (Vermette and Meagher 2003). Thus one of the main objectives of this chapter is to provide valuable information to bridge the knowledge gap between the theoretical and actual conformation of grafted PEGs and latter's dependence on grafting density and layer thickness. Finally, based on these surface quantification data, the nature of interactions between the matrix and protein are analysed in detail and their influence on the results of solid-phase PEGylation method are ascertained.

Table 5-1: Various microanalytical surface characterization techniques used and their properties (Miyoshi 2002)

Technique	Information	Resolution		Sample	Popularity
		Vertical	Lateral		
X-ray photoelectron spectroscopy (XPS)	Elemental composition, chemical state, depth profiling, imaging and mapping	A few to several nm	5 μm to 5 mm	Ultra high vacuum compatible solids	Extensive
Scanning electron microscopy (SEM)	Imaging, morphology, elemental composition, damages, defects, crystallography, grain structure, magnetic domain	Variable from a few nm to a few μm	1 to 50 nm in secondary electron mode	Conductors and coated insulators	Extensive
Confocal laser scanning microscopy (CLSM)	3D and 2D imaging, morphology, profilometry, topographic imaging, film thickness, wear volume, scar and crater depth, defects	Variable from a few nm to a few μm	0.5 to 4 μm (optical)	All	Medium

5.2 Microanalytical surface characterization tools

Microanalytical tools are characterised with their ability to identify, characterize and estimate the trace components and composition of solid surfaces down to extremely low concentrations. These techniques will provide valuable and in-depth information regarding the physical, chemical and mechanical properties of surfaces. Each technique will provide unique but often complementary information. Various surface analytical tools used in this study are summarized in Table 5-1.

5.2.1 X-ray photoelectron microscopy (XPS)

XPS, also known as electron spectroscopy for chemical analysis (ESCA), is a very powerful surface analytical tool with its characteristic elemental detection, chemical state identification and quantitative capabilities (de Vries 1998; Wolstenholme 1999). The basic principle behind XPS is a photoelectric effect, which involves the emission of electrons due to the absorption of X-rays (Figure 5-1).

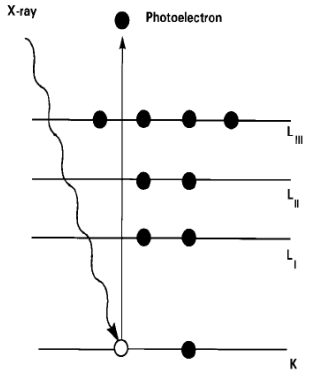


Figure 5-1: XPS principle (de Vries 1998)

In XPS, samples are irradiated with a monochromatic beam of X-rays with a known energy that penetrates through 1–10 μm into the sample surface, resulting in the emission of electrons. Due to very small difference in the mean-free paths, only the electrons emitted from the few top atomic layers are detected. The kinetic energy (E_k) of the emitted electrons will be

$$E_K = h\nu - E_B - w \qquad (5\text{-}1)$$

where hν is the energy of interacting photon, E_B is the binding energy of electron in the atomic orbital from which it originates and w is the work function of the spectrometer. Binding energy (E_B) can be interpreted as the ionization energy of a particular atom and hence it is characteristic of the atom in a particular orbital with a particular bonding environment. This results in a distinctive energy position and intensity distribution for a particular atom, enabling its identification and quantification.

5.2.1.1 Characterization and quantification of PEG grafted solid matrices using XPS

XPS survey scans are known to be very effective in characterizing PEG grafted onto various solid matrices such as silicon (Sofia et al. 1998; Popat and Desai 2004; Popat et al. 2004; Michel et al. 2005), polystyrene (Brindley et al. 1995; Cairns et al. 1999), nonporous alumina (Popat et al. 2004), other metal surfaces (Feller et al. 2005; Tanaka et al. 2007) and even functionalized nanoparticles (Pourcelle et al. 2007; Hu et al. 2008; Wang et al. 2008). The presence of PEG on a matrix surface can be characterised by a sharp increase in the C 1s and O 1s peaks at 285 eV and 528 eV, respectively. High resolution C 1s scans provide more in-depth information from the characteristic C-O peak, with its intensity

directly proportional to the PEG concentration on the surface. Popat and his co-workers correlated the relative intensities of the C-O (at 286.5 eV) and C-C (at 285 eV) peaks with PEG concentration using both Langmuir and exponential models (Popat et al. 2004). The relative ratio of oxygen to carbon (O/C) will vary with the amount of PEG attached to the solid surface, thus enabling the quantitative determination of PEG on the surface (Kingshott et al. 2002).

Thickness (t, nm) of the PEG layer can be calculated by measuring the electron attenuation length (E_L, nm) of the carbon peak, using Equation 5-2 (Sofia et al. 1998)

$$I = I_0 \left(\frac{t}{E_L . \sin\theta} - 1 \right) \tag{5-2}$$

where I_0 and I are the relative intensities (eV) of C-O peaks within the high resolution C 1s scans before and after PEG grafting and θ is the angle of incidence of the X-rays on the sample surface. Electron attenuation length of the carbon peak is characteristic of its electron energy (E) and can be calculated from Equation 5-3 (Tao et al. 2008),

$$E_L = \frac{49}{E^2 \rho} + 0.11 \frac{\sqrt{E}}{\rho} \tag{5-3}$$

where ρ is the density of PEG (1.1 g/cm^3) and the electron energy E can be given as

$$\begin{aligned} E = & \text{ (X-ray core energy – core binding energy of C)} \\ = & \text{ (1486 – 285) = 1201 eV.} \end{aligned} \tag{5-4}$$

Knowing the thickness, the surface concentration (Γ, g/nm^2) and the average distance between grafted PEG chains (*D*, nm) can be calculated using Equations 5-5 and 5-6, respectively (Tao et al. 2008)

$$\Gamma = \rho\, t \tag{5-5}$$

$$D = \left(\frac{M}{\Gamma N_A} \right)^{1/2} \tag{5-6}$$

where M is PEG molecular weight (Da) and N_A is Avogadro's number (6.023 x 10^{23} mol^{-1}). Finally, the grafting density (σ) (de Gennes 1980) can be calculated from Equation 5-7,

$$\sigma = \left(\frac{a}{D} \right)^2 \tag{5-7}$$

where *a* is the size of a PEG monomer unit.

5.2.1.2 Conformational studies of grafted PEG molecules using XPS

XPS core line and valence band spectra are sensitive to changes in polymer chain conformations due to their influence in the valance molecular orbitals. Beamson and his co-workers found that the XPS valance band spectra for PEG constitutes two distinguishing peaks, named "a" and "b" (Figure 5-2), representing antibonding and bonding molecular orbitals, in the valance band region of the spectrum (Beamson et al. 2000).

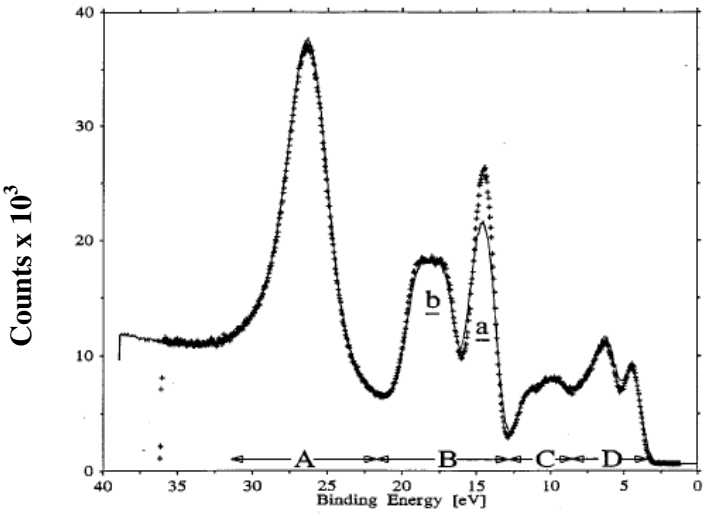


Figure 5-2: Valence band spectra of PEG [Region A,B,C and D corresponding to ionization from MOs consisting largely of O 2s, (C 2s + O 2s), (C 2p + O 2s + H 1s) and (O 2s + O 2p] (Beamson et al. 2000)

In the case of PEG chains with a brush conformation, there is a stabilization of the molecular orbitals (MOs) through methylene hyperconjugation between the alternate carbon atoms, resulting in a crowding of energy levels above the C 2s orbitals, which produces a sharp antibonding peak in the spectrum (peak "a"). In contrast, as in the mushroom conformation with random coils, stabilization through hyperconjugation is significantly reduced, resulting in a dispersion of energy levels above the MOs. This results in a reduction in the height of peak "a". Thus, comparing the relative heights of these antibonding and bonding peaks provides a useful tool in studying the conformational changes of PEG polymers.

Because the sensitivity for detecting local conformational changes with XPS can be extended only for a few repeating units and also because of the very small effective core level and valence band shifts induced by conformational changes, highly sensitive and accurate experimentation and instrumental performances are required to characterize these changes.

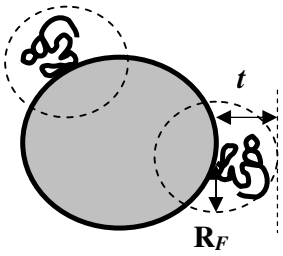
For these reasons, despite the fact that Beamson and his colleagues (Beamson et al. 2000; Beamson 2007) could detect conformational changes of pure PEG molecules using this methodology, its sensitivity for distinguishing various conformational isomers of PEG grafted onto a solid matrix is doubtful.

In an alternative and simplified manner, the thickness of a grafted PEG layer can be correlated with its conformation behaviour (Su et al. 1997). With a lower thickness, PEG molecules will be predominantly in the mushroom conformation, whereas a higher thickness indicates stretching out of the PEG molecule as polymer brushes. As a corollary, the conformational properties of a tethered PEG molecule at the surface can be precisely predicted by comparing the distance between the grafted PEG chains with the Flory radius (R_F) of the polymer. R_F can be calculated knowing the size and the number (N) of its monomer units (Meng et al. 2004a).

$$R_F = a\ N^{3/5} \qquad (5\text{-}8)$$

In the case of PEGs with a mushroom conformation, D will be greater than R_F and the thickness of the PEG layer will be equal to R_F (Figure 5-3a). However, if the PEG polymer exists in its brush form, D will be smaller than R_F and the thickness will be greater than R_F (Figure 5-3b). Although the transformation of mushroom to brush conformation begins immediately once the distance between grafted PEG chains increases above R_F, brush forms will not be fully developed until the surface concentration is sufficiently large to have a very compact packing of the grafted molecules (Hansen et al. 2003). Taking this into consideration, assignment of a brush conformation is proposed only when the layer thickness is significantly larger than the R_F values, say when $t > 2R_F$.

a)



b)

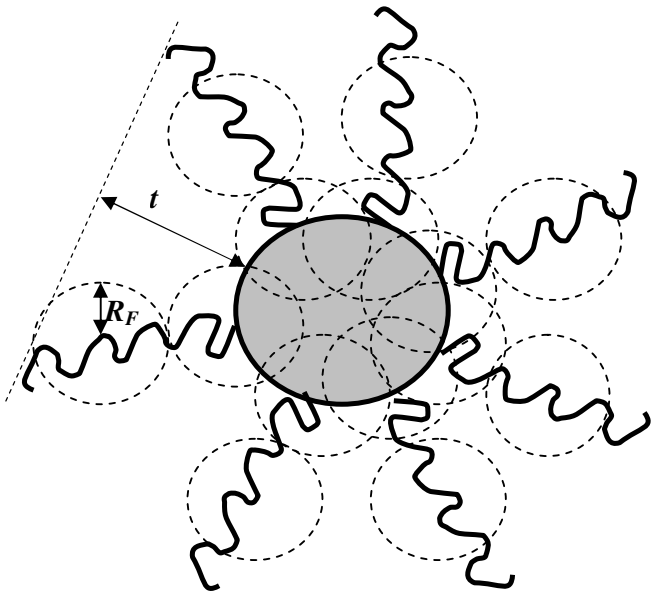


Figure 5-3: a) PEG with mushroom conformation and b) PEG with brush conformation (Not to scale)

Thus, a detailed comparison of the three parameters *D*, R_F and *t*, will provide a useful tool in distinguishing the possible PEG conformations on the solid interface. Based on these, the following hypothesis can be derived to distinguish the various conformers as summarized in Table 5-2.

Table 5-2: Hypotheses to distinguish the PEG conformations

Hypothesis	Conditions (and / or)	Conformation
A	$D > R_F$	Mushroom
	$t \leq R_F$	
B	$R_F < t < 2R_F$	Intermediate
C	$D < R_F$	Brush
	$t > 2R_F$	

5.2.1.3 Comparison with literature

In the absence of a detailed PEG conformational assignment based on XPS data, the correlation between PEG layer thickness and conformational behaviour of PEGs reported in other literature was considered to validate the methodology. Comparing the results, summarized in Table 5-3, a brush conformation is assigned generally when $t > 2R_F$. Various methods were adopted for these conformational assignments such as force measurements (Xu and Marchant 2000; Heuberger et al. 2005; Nnebe and Schneider 2006), changes in ζ-potential (Meng et al. 2004a) and use of ellipsometry (Zdyrko et al. 2004; Zdyrko et al. 2009) indicates a generalized validity of the concept and hypothesis.

Table 5-3: Conformational assignment from literature and its match with the hypothesis

PEG MW	*t* nm	*D* nm	R_F nm	Confo-rmation	Hypo-thesis	Reference
2000	7	--	3.43	Brush	C	(Xu and Marchant 2000)
2000	8.2	1.4	3.43	Brush	C	(Heuberger et al. 2005)

Table 5-3 continued

3400	9.55	1.66	4.74	Brush	C	(Meng et al. 2004a)
5000	11.5	0.9	5.96	Brush	C	(Zdyrko et al. 2004)
5000	13	--	5.96	Brush	C	(Nnebe and Schneider 2006)
5000	7 to 9	--	5.96	Interm-ediate	B	(Zdyrko et al. 2009)

5.2.1.4 Study of protein interaction with PEG grafted matrices using XPS data

According to de Gennes (1987), interactive forces exerted between two surfaces having end grafted polymers is a function of polymer grafting density and distance between the two surfaces (Equation 2-1). Kim and Luckham extended this equation to predict the force of interaction between two spherical matrices bearing polymeric chains and is given by Equation 5-9 (Kim and Luckham 1992),

$$F = \pi r\left(\frac{\beta kT}{D^3}\right)\left(\left\{\left[\frac{(2t)^{2.25}}{0.3125}\frac{1}{h^{0.25}} - \frac{h^{2.75}}{4.8125(2t)^{0.75}}\right] + \left(\frac{8t}{5}h + \frac{8t}{7}h\right)\right\} - \left[4.3636(2t)^2\right]\right) \qquad (5\text{-}9)$$

where r is the matrix radius and β is a constant (β = 12 (Kim and Luckham 1992)). Thus, considering aqueous solutions of globular proteins as colloidal spheres, this equation can be extended to measure the interactive forces by knowing the PEG grafting profile and the distance between the approaching protein and matrix.

5.2.2 Scanning electron microscopy (SEM)

SEM is a very powerful imaging technique characterised by its capability of producing detailed surface morphological and topological informations. In SEM, a stream of monochromatic electrons produced by an electron gun are focused through a fine probe and scanned over a small rectangular area of the sample specimen (Flegler et al. 1993; Vernon-Parry 2000). Interaction of electron beam with the specimen generates various signals such as secondary, back scattered, photon emission etc which are detected by appropriate detectors in the specimen chamber, based on their varying level of energies. The secondary electrons produced within the first few nanometres of the sample surface, having an average energy less between 3 to 5 eV, provide the highest spatial resolution images and help in studying the useful topographic informations.

5.2.3 Confocal laser scanning microscopy (CLSM)

Confocal microscopy, based on the concept developed by Minsky (1988), is characterized by point-wise illumination and detection. Its unique capability for eliminating scattered and reflected lights from out of focus planes yields images with an improved resolution and contrast over other conventional microscopes (Miyoshi 2002).

The principle of confocal microscopy is illustrated in Figure 5-4 (Prasad et al. 2007). With the help of a dichromatic mirror and a pair of rotating mirrors, laser light is directed through the microscope object and excites the fluorescent sample. The reflected fluoresced light from the sample is then allowed to pass through the dichromic mirror after descanning by the same pair of rotating mirrors.

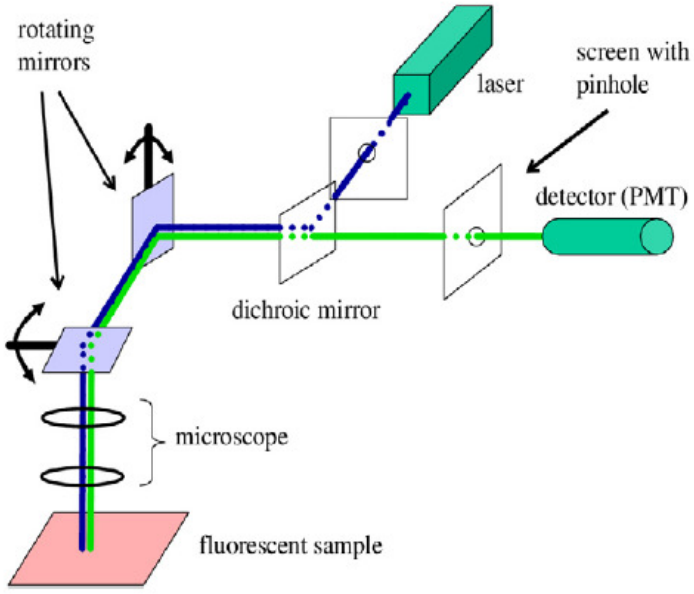


Figure 5-4: Schematic representation of confocal microscope (Prasad et al. 2007).

Light coming from the dichromic mirror is then passed through a pinhole placed in the conjugate focal plane of the sample, which rejects all out of focus light. Finally, these rays are recorded by a photomultiplier detection tube without scattering in the lateral resolution. The capability for reconstructing a three dimensional image of the sample by combining a series of two dimensional image planes is one of the main advantages of CLSM over other microscopic techniques.

The core importance of confocal microscopy in this context is to make use of its special capability for fluorescent imaging (Pygall et al. 2007) with its high sensitivity and low intrinsic backgrounds. Fluorescent imaging of fluorescein isothiocyanate (FITC) labelled Cytochrome c conjugated solid matrixes were recorded to confirm any conjugation of the protein on the matrix.

5.3 Methods

5.3.1 XPS

PEG grafted solid matrices were analysed by XPS to characterise and quantify the PEG concentrations and to study conformational properties. PEG grafted samples were prepared with polystyrene, CM Sephadex and CP Sephadex, following the method given in Section 4.5.2. In general, samples grafted at 40°C with a grafting time of 12, 24, 48 and 72 hour were taken for XPS characterizations. In order to study the influence of temperature, PEG-grafted polystyrene and CP Sephadex were also prepared at 25 and 55°C with a 72 hour grafting period. Hereinafter, PEG grafted samples are abbreviated by matrix, PEG MW, reaction time and temperature. For example, PS200012H40C, denotes a polystyrene matrix, 2000 molecular weight PEG, a 12 hour grafting time and 40°C grafting temperature.

XPS analysis was performed on a PHI-5800 spectrometer (Physical Electronics, MN, USA) (Figure 5-5). Analyses were performed using a monochromatic Al Kα X-ray source (1486.6 eV) with an Omni Focus III small area lens and multichannel detector. Measurements were taken at an electron take-off angle of 45° (θ = 45°) from a normal sampling surface depth of ~50 Å. The spectrometer samples a surface area of approximately 400 μm by 700 μm and a concentric hemispherical analyser, which operates in the constant analyzer transmission mode, measures the binding energies of the emitted photo electrons. The binding energy scale was calibrated prior to analysis by the $Au4f_{7/2}$ peak at 83.9 eV and linearity was verified by the $Cu3p_{1/2}$ and $Cu2p_{3/2}$ peaks at 76.5 and 932.5 eV, respectively.

A uniform, thin layer of samples was prepared on a double-sided adhesive graphite tape and degassed at 10^{-7} to 10^{-9} mbar vacuum for approximately one hour before introducing to the XPS stage. Survey scans were collected from 10 to 1100 eV with a pass energy of 187.85 eV. All spectra were referenced by setting the C1s peak to 285.0 eV to compensate for residual charging effects. High resolution spectra for C1s were recorded between 282.0 eV and 294.0 eV, with a pass energy of 23.50 eV. The peak areas were quantified using a Gaussian-Lorentzian fitting procedure using the XPSPeak 4.1 software provided with the instrument.

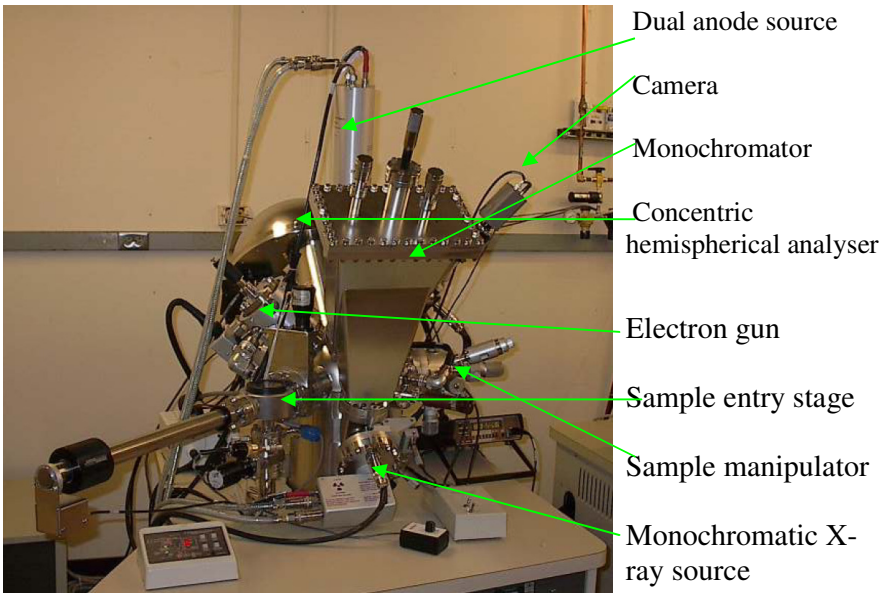


Figure 5-5: PHI-5800 spectrometer and its components

5.3.2 SEM

The surface morphologies of Sephadex and polystyrene derivatives before and after PEG grafting were examined by SEM using a Leica Cambridge S440 Scanning Electron Microscope (Leica, Rockleigh, NJ, USA) operating at an acceleration voltage of 5.0 kV with a probe current

of 50 Pa and a working distance of10 mm. All samples were sputter coated with 100 Å Gold thicknesses using a sputter-coater (EMITECH K550X, Emitech, Ltd., Ashford, UK) for 2 minutes at 20 mA with a high tension 1.2 kV.

5.3.3 Fluorescent imaging by CLSM

5.3.3.1 FITC labelling of Cytochrome c and its conjugation with NHS activated PEG resins.

Labelling of Cytochrome c with fluorescein isothiocyanate (FITC, Sigma-Aldrich) was carried out based on the methodology reported by Zalk et al. (2005). To a solution of Cytochrome c (2 mg/mL) in 100 mM carbonate buffer (pH 9.0), 1 mg/mL FITC in DMSO solution (50 μL FITC solution for 1 mL of Cytochrome c solution) was added very slowly under gentle stirring. After completing the addition of FITC solution, the resulting mass was stirred at 30°C for 2 hour. Unreacted FITC was separated from the resulting mixture by overnight dialysis (Dialysis buffer: 50 mM KCl, 5 mM $MgCl_2$, 5 mM $NaHCO_3$ and 20 mM Hepes, pH 7.35) with multiple change of dialysis buffer. The ratio of FITC to Cytochrome c was estimated by measuring the absorbance at 495 nm and 280 nm and found to be 2.35 ± 0.19.

Carboxymethyl Sephadex, carboxypentyl Sephadex and carboxylated polystyrene solid matrices were grafted with the α-(β-alanine)-ω-carboxy PEG derivative (**6**), followed by ethanolamine blocking. The free carboxy terminal of the tethered PEG was then further activated with NHS ester and treated with FITC labelled Cytochrome c. Cytochrome c conjugated matrices were then washed thoroughly with PBS buffer (x2), 2 M NaCl (x2) and PBS buffer (x2).

5.3.3.2 CLSM

Fluorescence released from the FITC labelled Cytochrome c was analyzed with Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 63×planapo objective (numerical aperture 1.4). A Kr/Ar laser was tuned to produce a 488 nm beam and images were recorded with a line average of 6 images.

5.4 Results and discussion

5.4.1 XPS – Surface characterization and conformational studies

Survey scans were taken to determine the elemental surface composition and high resolution C1s scans were performed to study the PEG chemistry in detail.

5.4.1.1 Survey scans

Survey scans were characterized by distinctive peaks for O1s at 528 eV and C1s at 285 eV with a minor peak at 400 eV corresponding to N1s (Figure 5-6). The relative composition of O and C atoms for various PEG grafted matrices are summarized from their survey scans and given in Table 5-4 as their oxygen to carbon (O/C) ratios. As the PEG concentration increases, the relative O/C ratio decreases for both PEG molecular weights. This decrease in O/C ratio with increasing PEG concentration matches well with the theoretical expectation and serves as very clear evidence for the presence of PEG and increasing grafting densities on these matrices. Because all the matrices under investigation were abundant with residual C and O atoms, it was difficult to extract quantitative information regarding PEG grafting from these survey scans alone. Therefore, high resolution C 1s scans were performed to provide

more precise information regarding PEG grafting chemistry and conformational behaviour. A summary of survey scans for various PEG grafted polystyrene, CM Sephadex and CP Sephadex matrices are given in Appendix B (Figure B-1 to B-6).

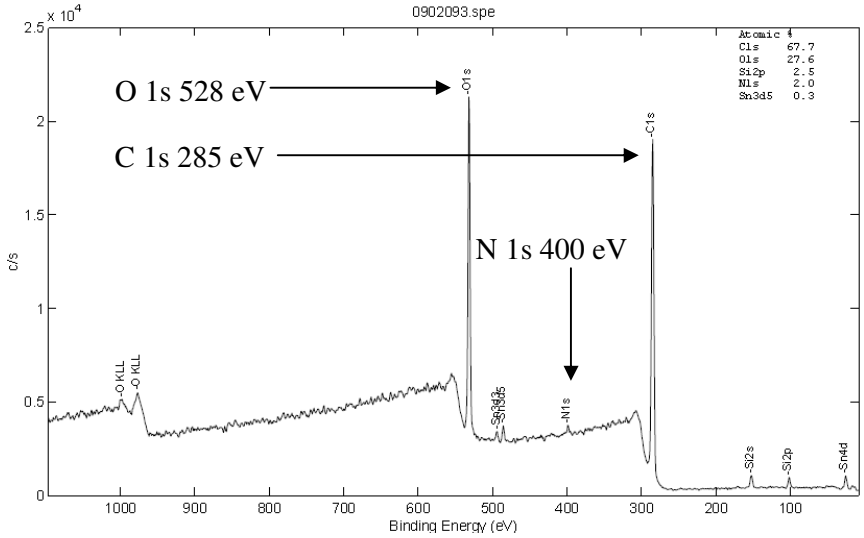


Figure 5-6: XPS survey scans (PS400012H40C)

Table 5-4: Surface O/C ratio for various PEG grafted matrices

PEG matrix	**O/C ratio for PEG grafting parameters (Time (hr) / Temperature (°C))**					
	12/40	**24/40**	**48/40**	**72/40**	**72/25**	**72/55**
PS2000	0.26	0.24	0.18	0.15	--	--
PS4000	0.41	0.36	0.33	0.33	0.41	0.34
CMSP2000	0.74	0.72	0.69	0.68	--	--
CMSP4000	0.73	0.71	0.71	0.70	--	--
CPSP2000	0.62	0.57	0.56	0.56	--	--
CPSP4000	0.62	0.61	0.59	0.54	0.61	0.52

5.4.1.2 High resolution C 1s scans

High resolution C 1s scans can be resolved into various components, corresponding to C-C and C-H bonds at 285.0 eV, C-O bond at 286.5 eV, >C=O bond at 288.0 eV and $–COO^-$ and –NH-CO- bonds at 289.0 eV (Nakao et al. 1996; Zhang et al. 2006). In the case of PEG grafted solid matrices and surfaces, there will be a substantial increase in the intensity of the C-O peak at 286.5 eV with increasing PEG concentration, which is a characteristic indication of PEG coupling (Popat et al. 2004) (Figure 5-7).

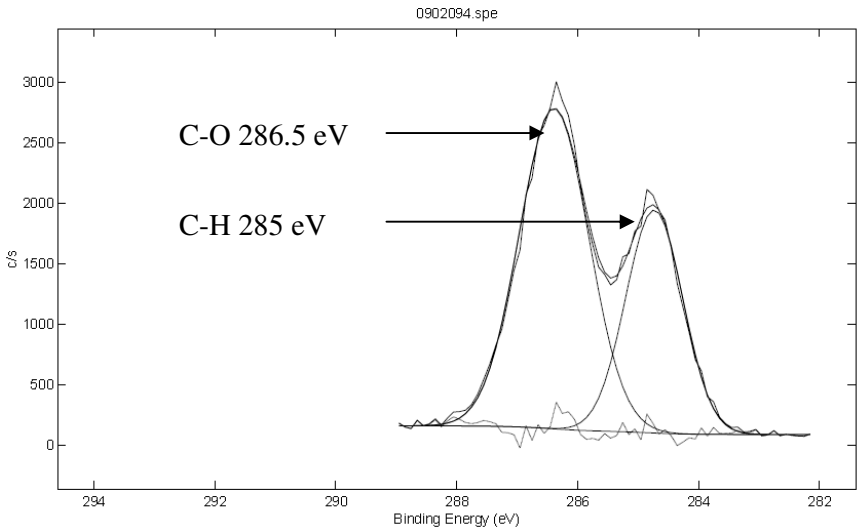


Figure 5-7: XPS high resolution C 1s scans (PS400072H40C)

The relative percentages of the C-O peak in the overall C 1s high resolution scans were calculated with the help of XPSPeak 4.1 software provided with the instrument and were used as the measure of PEG grafting profile. Peak areas were quantified with a Gaussian-Lorentzian fitting procedure for the C-O peak and this was, in turn, used for further estimation of PEG and its conformation. The choice of C-O peak intensities exclusively for these calculations was based on its direct

correlation with the PEG concentration, as reported in the literature (Popat et al. 2004; Popat et al. 2004; Sharma et al. 2004; Tao et al. 2008) and also due its compatibility with the standard uniform overlay model given by Equation 5-2 (Sofia et al. 1998).

(Note: Overlay of C 1s high resolution scans obtained for various PEG grafted matrices under different grafting conditions are summarized in Appendix B (Figure B-7 to B-14).

5.4.1.3 Characterization and quantification of grafted PEG

The thickness of the PEG layer was calculated using Equation 5-2, knowing intensities of the matrix before and after PEG grafting. In the case of PEG grafted polystyrene samples, the intensity of C-O peak for the unmodified polystyrene matrix was taken as the initial intensity and compared with that of each PEG grafted matrix to calculate the layer thickness. Similarly, for CM Sephadex and CP Sephadex derivatives, C-O intensities from the base, unmodified matrices were taken as the initial intensities for calculating PEG thickness for the respective PEG modified matrices.

When PEG thickness was calculated using Equation 5-2, a residual thickness of 4.901 nm was observed when $I = I_0$. This arbitrary residual thickness is a characteristic of a particular instrument, peak and electron take-off angle and was subtracted from the calculated values to obtain the absolute thickness. By knowing the PEG layer thickness, other important quantification parameters, such as surface concentration and grafting densities, were calculated using Equations 5-5 and 5-7, respectively. A summary of these results is given in Tables 5-5 to 5-7.

Table 5-5: Surface characterization of PEG grafted polystyrene matrices

Matrix	I_0	I	PEG quantification		
	eV	eV	Thickness *t*, nm	Surface concentration Γ g/nm^2	Grafting density σ
PSCOOH	652				
PS200012H40C		1414	2.86	3.15 x10^{-21}	0.116
PS200024H40C		1498	3.18	3.50 x10^{-21}	0.129
PS200048H40C		1655	3.77	4.15 x10^{-21}	0.153
PS200072H40C		2059	5.29	5.82 x10^{-21}	0.215
PS400012H40C		2139	5.59	6.15 x10^{-21}	0.113
PS400024H40C		2405	6.59	7.25 x10^{-21}	0.134
PS400048H40C		2991	8.79	9.67 x10^{-21}	0.178
PS400072H40C		3811	11.87	1.31 x10^{-20}	0.241
PS400072H25C		3.22	8.91	9.80 x10^{-21}	0.181
PS400072H55C		4323	13.80	1.52 x10^{-20}	0.280

Table 5-6: Surface characterization of PEG grafted CM Sephadex matrices

Matrix	I_0	I	PEG quantification		
	eV	eV	Thickness *t*, nm	Surface concentration Γ g/nm^2	Grafting density σ
CM Sephadex	351				
CMSP200012H40C		347	0	0	0
CMSP200024H40C		363	0.08	9.22 x10^{-23}	0.003
CMSP200048H40C		406	0.38	4.22 x10^{-22}	0.016
CMSP200072H40C		408	0.40	4.38 x10^{-22}	0.016
CMSP400012H40C		351	0	0	0

Table 5-6 continued

CMSP400024H40C	457	0.74	8.14 x10^{-22}	0.015
CMSP400048H40C	554	1.42	1.56 x10^{-21}	0.029
CMSP400072H40C	727	2.62	2.89 x10^{-21}	0.053

Table 5-7: Surface characterization of PEG grafted CP Sephadex matrices

Matrix	**I_0**	**I**	**PEG quantification**		
	eV	**eV**	**Thickness *t*, nm**	**Surface concentration Γ g/nm^2**	**Grafting density σ**
CP Sephadex	720				
CPSP200012H40C		692	0	0	0
CPSP200024H40C		873	0.52	5.73 x10^{-22}	0.021
CPSP200048H40C		1067	1.18	1.30 x10^{-21}	0.048
CPSP200072H40C		1485	2.60	2.86 x10^{-21}	0.106
CPSP400012H40C		1031	1.06	1.16 x10^{-21}	0.022
CPSP400024H40C		1474	2.57	2.82 x10^{-21}	0.052
CPSP400048H40C		1814	3.72	4.10 x10^{-21}	0.075
CPSP400072H40C		1936	4.14	4.55 x10^{-21}	0.084
CPSP400072H25C		856	0.46	5.09 x10^{-22}	0.009
CPSP400072H55C		1642	3.14	3.45 x10^{-21}	0.064

Only a limited number of samples were repeated to check the accuracy of the analysis. A summary of the results obtained from repeat analysis are given in Table 5-8 and were found to be fairly consistent with the initial results. These results provided a convincing proof for the fair accuracy of the entire results in absence of an elaborate error analysis of the entire samples due to the analysis constraints.

Table 5-8: XPS standard deviations

Matrix	I eV	PEG Thickness *t*, nm	SD	% SD
PS400072H40C	3811	11.878		
PS400072H40C (R)	3953	12.41	0.38	3.11
CMSP400072H40C	727	2.63		
CMSP400072H40C (R)	674	2.26	0.26	10.72
CPSP400072H40C	1936	4.14		
CPSP400072H40C (R)	2016	4.41	0.19	4.50

Figures 5-8 and 5-9 illustrate the grafting profile for these matrices. Analyzing these results, higher PEG grafting was observed on polystyrene matrices than either of the Sephadex derivatives with both PEG molecular weights. The likely reason for this is the difference in swelling behaviour of these matrices in the anhydrous reaction environments where the PEG grafting was performed. Polystyrene matrices swelled significantly in anhydrous organic solvents, whereas the Sephadex matrices remained unswelled, leaving fewer active sites for PEG conjugation.

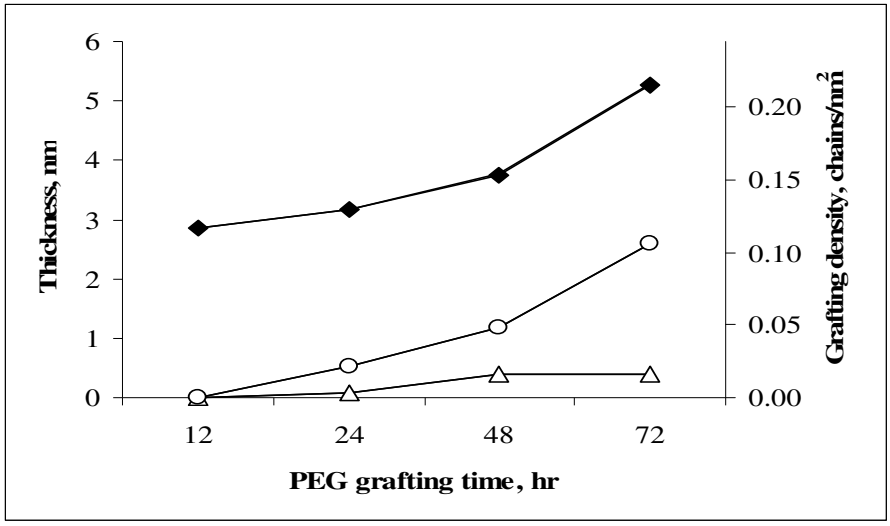


Figure 5-8: Grafting profile for PEG2000 at 40°C (—◆—PS2000, —○—CP2000 and —△—CM2000)

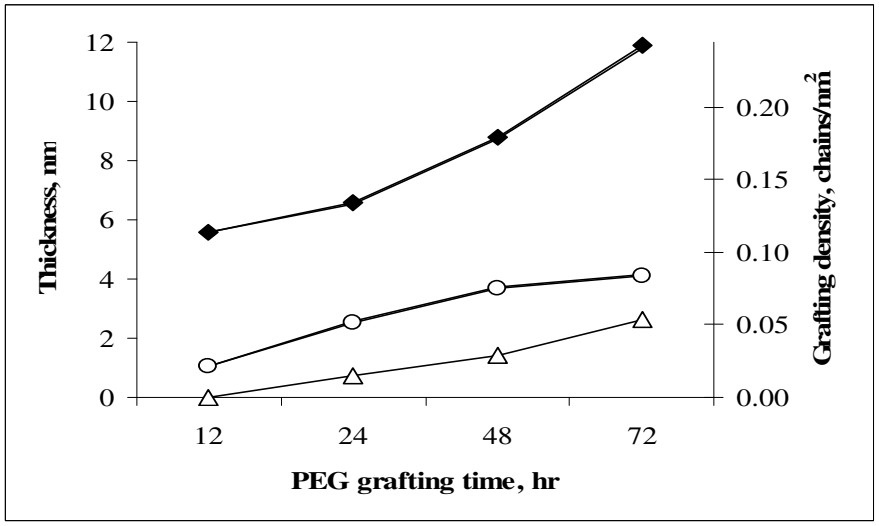


Figure 5-9: Grafting profile for PEG4000 at 40°C (—◆—PS4000, —○—CP4000 and —△—CM4000)

In the case of Sephadex derivatives, the chain length of the carboxyl tether greatly influenced PEG grafting density. The presence of a relatively long carboxypentyl tether resulted in comparatively less steric crowding during PEG grafting compared with that of its shorter

carboxymethyl analogue. Consequently, slightly higher PEG grafting concentrations were achieved with the CP Sephadex derivative than with its CM analogue under similar grafting conditions. The thickness of PEG layers obtained with 4000 Da PEG were found to be almost double that obtained with 2000 Da PEG for all matrices at each grafting condition. PEG molecular weight therefore influenced layer thickness, irrespective of the nature of matrix used.

In order to study the influence of temperature on PEG grafting kinetics, polystyrene and CP Sephadex derivatives were conjugated with 4000 Da PEG for 72 hour at 25°C, 40°C and 55°C. The impact of temperature on the layer thickness and grafting density is illustrated in Figure 5-10, showing a substantial increase up to 40°C. However, on further increases in temperature, a decrease in the PEG surface concentration was observed, due to an increase in the competing hydrolysis reaction of the NHS active ester, resulting in less available sites for conjugation.

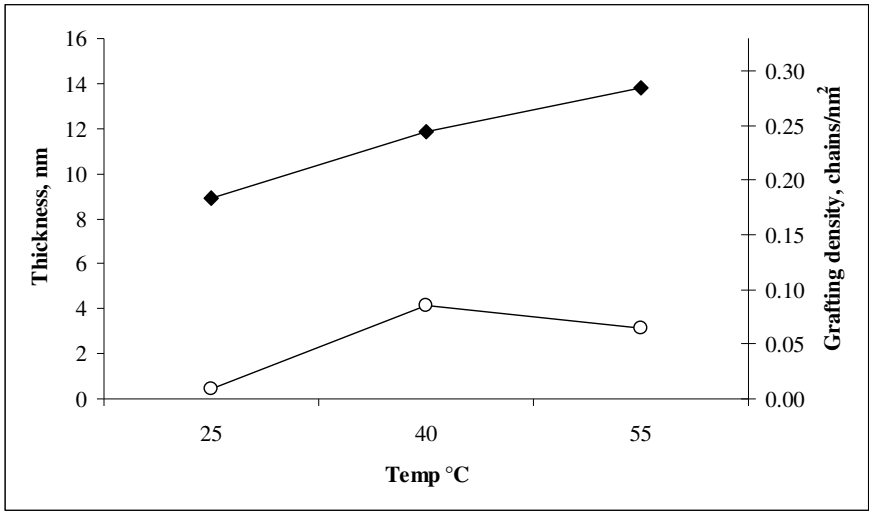


Figure 5-10: Influence of temperature on grafting profile (Parameters: PEG MW: 4000 and grafting time 72 hr) (—◆—PS4000 and —○—CP4000)

5.4.1.4 Conformational studies of the grafted PEG

Although XPS has been proven to be a very powerful tool for characterization and quantification of surface grafted PEG molecules, only limited efforts have been reported to date to understand the conformational behaviour of the grafted molecules. Using the proposed hypothesis (Table 5-2), a detailed study into the conformational behaviour of grafted PEG layers was initiated. The present hypothesis correlates the conformational properties of the grafted PEG molecules with its Flory radius, distance between the grafted molecules and thickness of the polymer layer on the matrix.

Flory radii of the PEG chains were calculated using Equation 5-8. In this case, PEGs with molecular weights 2000 Da and 4000 Da were used, which have a numbers of monomer units N = 45 and 90, respectively, and the size of the monomer unit is 0.35 nm (Hansen et al. 2003). Therefore, R_F values for PEG 2000 Da and 4000 Da will be equal to 3.435 nm and 5.207 nm, respectively. Knowing these R_F values, the distance between the PEG molecules on various solid matrices under investigation were calculated using Equation 5-6. Finally thickness, distance between the PEG molecules and Flory radius were correlated with hypotheses A, B and C in Table 5-2 to assign the possible conformations for the grafted polymer. Conformational assignments for various PEG grafted polystyrene matrices are summarized in Table 5-9.

Table 5-9: Conformational properties of PEG grafted polystyrene matrices

PEG Matrix	*t*, nm	*D*, nm	R_F, nm	Hypothesis	Conformation
PS200012H40C	2.86	1.27	3.44	A	Mushroom
PS200024H40C	3.18	0.97		A	Mushroom
PS200048H40C	3.77	0.89		B	Intermediate
PS200072H40C	5.29	0.76		B	Intermediate
PS400012H40C	5.59	1.04	5.21	B	Intermediate
PS400024H40C	6.59	0.96		B	Intermediate
PS400048H40C	8.79	0.83		B	Intermediate
PS400072H40C	11.87	0.71		C	Brush
PS400072H25C	8.91	0.82		B	Intermediate
PS400072H55C	13.80	0.66		C	Brush

With both 2000 and 4000 Da PEGs, the layer thickness increases with reaction time, with a simultaneous decrease in distance between the grafted PEG molecules (Figure 5-11). This indicates a more compact grafting profile on the surface. Applying the proposed hypothesis for conformational assignments to these samples was quite straight forward, in that the thickness of the PEG layers on the matrices can be easily correlated with the Flory radius of the PEG chains. In the case of polystyrene, grafted with 2000 Da PEG, the thickness of the PEG layers obtained after 12 and 24 hour grafting at 40°C were well below the R_F value. These values were clearly in accordance with the criteria of hypothesis A and the mushroom conformation can therefore be assigned for these grafted PEG molecules. As the grafting time increased, the layer thickness increased above the R_F value, thus changing to a brush-mushroom intermediate conformation following hypothesis B.

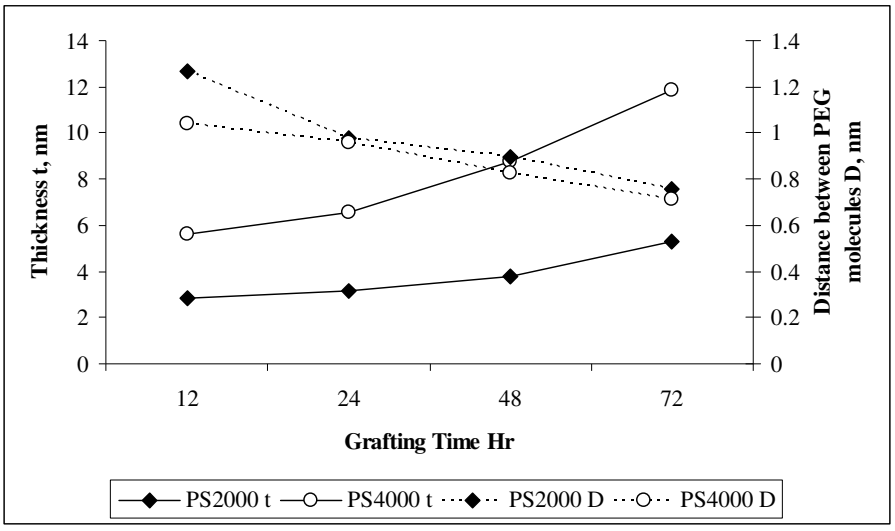


Figure 5-11: Relationship between PEG layer thickness and distance between the PEG molecules with PEG grafted polystyrene matrices (at 40°C)

With 4000 Da PEG, the layer thickness on the polystyrene matrices was greater than R_F even after 12 hour of grafting (at 40°C), resulting in an intermediate brush-mushroom conformation for these samples. The thickness of the matrix obtained after 72 hour grafting at the same temperature was much higher ($t > 2R_F$), indicating a brush conformation for its grafted PEG molecules (hypothesis C). Hypothesis C is undoubtedly validated under this highest grafting density condition resulting in the smallest distance between the grafted PEG chains ($D << R_F$). This indicates that polymer chains were stretched out from the matrix surface to accommodate more PEG molecules, resulting in the formation of polymer brushes on the surface.

To support these results, with independent observations, layer thickness and conformation of grafted PEGs summarized in Table 5-3 can be compared with the present results. Meng et al. (2004a) reported a layer thickness of 9.55 nm and surface concentration of 2.04 x 10^{-21} g/nm^2 for a

3400 Da PEG grafted on polystyrene matrices for a brush conformation. This surface concentration was measured by an indirect assay method by estimating the amine content, based on UV absorption. Thus, assigning a brush conformation for PEG 4000 Da grafted surfaces with a layer thickness of 11.87 nm and above appears to be justified within the experimental errors and uncertainties. In many cases conformational assignments were performed based only on measuring the layer thickness, or the distance between the polymer chains were measured independently using another instrument and compared with the results. One of the advantages of XPS over these methodologies is their inherent capability to measure both the layer thickness and the distance simultaneously making the results and predictions more accurately than other methods.

The influence of reaction temperature on the grafted PEG conformation is clearly demonstrated with a change in conformation from an intermediate brush-mushroom to the brush form with an increase in temperature from 25°C to 55°C at a given grafting period. This change in conformation is manifested by an increase in the PEG layer thickness, followed by a reduced distance between the grafted PEG chains (Figure 5-12).

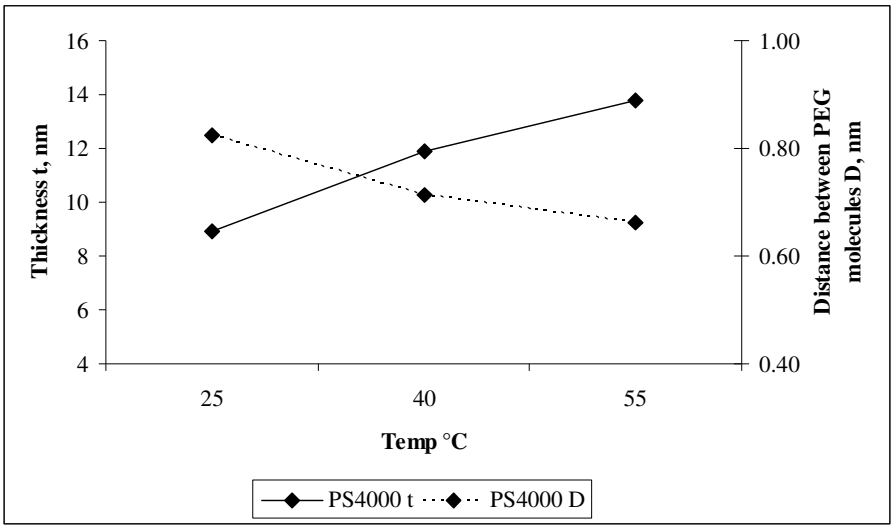


Figure 5-12: Influence of temperature on PEG layer thickness and distance between the PEG molecules with PEG grafted polystyrene matrices (at 72 hour)

With CM Sephadex (Table 5-10) and CP Sephadex derivatives (Table 5-11), the thickness of PEG layers obtained with both 2000 and 4000 Da PEGs were well below their R_F values, indicating the presence of mushroom conformations under all grafting conditions. The PEG grafted matrix obtained with 2000 Da PEG after 24 hour shows a very large distance between the PEG molecules compared to R_F, further validating hypothesis A to confirm the existence of a mushroom conformation with these grafted PEG molecules.

The influence of temperature on the grafting kinetics does not show any predominant conformational changes in the case of PEG grafted CP Sephadex derivatives, as it could impart only small changes in the layer thickness and grafting density (Table 5-11). Consequently, the grafted PEG molecules on CP Sephadex derivatives remained in their mushroom conformations even when grafting was carried out at higher temperatures.

Table 5-10: Conformational properties of PEG grafted CM Sephadex matrices

PEG Matrix	*t*, nm	*D*, nm	R_F, nm	Hypo-thesis	Conform-ation
CMSP200012H40C	0	0	3.44	-	-
CMSP200024H40C	0.08	6.00		A	Mushroom
CMSP200048H40C	0.38	2.80		A	Mushroom
CMSP200072H40C	0.40	2.75		A	Mushroom
CMSP400012H40C	0	0	5.21	-	-
CMSP400024H40C	0.74	2.86		A	Mushroom
CMSP400048H40C	1.42	2.06		A	Mushroom
CMSP400072H40C	2.62	1.52		A	Mushroom

Table 5-11: Conformational properties of PEG grafted CP Sephadex matrices

PEG Matrix	*t*, nm	*D*, nm	R_F, nm	Hypo-thesis	Conform-ation
CPSP200012H40C	0	0	3.44	-	-
CPSP200024H40C	0.52	2.41		A	Mushroom
CPSP200048H40C	1.18	1.60		A	Mushroom
CPSP200072H40C	2.60	1.08		A	Mushroom
CPSP400012H40C	1.06	2.39	5.21	A	Mushroom
CPSP400024H40C	2.57	1.53		A	Mushroom
CPSP400048H40C	3.72	1.27		A	Mushroom
CPSP400072H40C	4.14	1.21		A	Mushroom
CPSP400072H25C	0.46	3.61		A	Mushroom
CPSP400072H55C	3.14	1.39		A	Mushroom

The relationship between the layer thickness and the distance between grafted PEG molecules shows a very similar trend to that of polystyrene derivatives and is summarized in Figure 5-13. As the grafting density increased, layer thickness also increased, with a decrease in distance between the grafted PEGs to accommodate more molecules on the matrix.

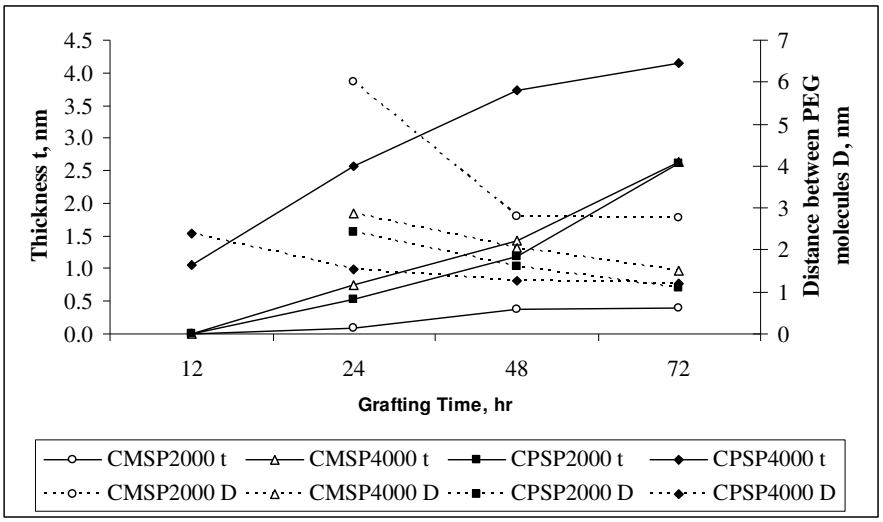


Figure 5-13: Relationship between PEG layer thickness and distance between the PEG molecules with PEG grafted CM and CP Sephadex matrices (at 40°C)

5.4.1.5 Force of interaction between protein and PEG grafted matrix

Interactive forces exerted between PEG grafted matrices and approaching proteins during conjugation can be studied by following the equation derived by Kim and Luckham (1992) (Equation 5-9), assuming both PEG grafted matrices and proteins were in spherical nature while in aqueous solution during conjugation. In order to evaluate the magnitude of these forces, a hypothetical case was considered in which the proteins were in the secondary potential energy minima (Figure 2-26) close to the end terminal of grafted PEG molecules. Consequently, the distance between

the matrix and protein will be equivalent to the PEG layer thickness ($h = t$, Equation 5-9). Applying this to Equation 5-9, the equation can be rearranged as

$$F = \pi r\left(\frac{\beta kT}{D^3}\right)\ 0.3849t^2 \qquad (5\text{-}10)$$

The force of interaction between approaching protein and various PEG grafted matrices were calculated using Equation 5-10 and is illustrated in Figure 5-14 as a function of PEG layer thickness.

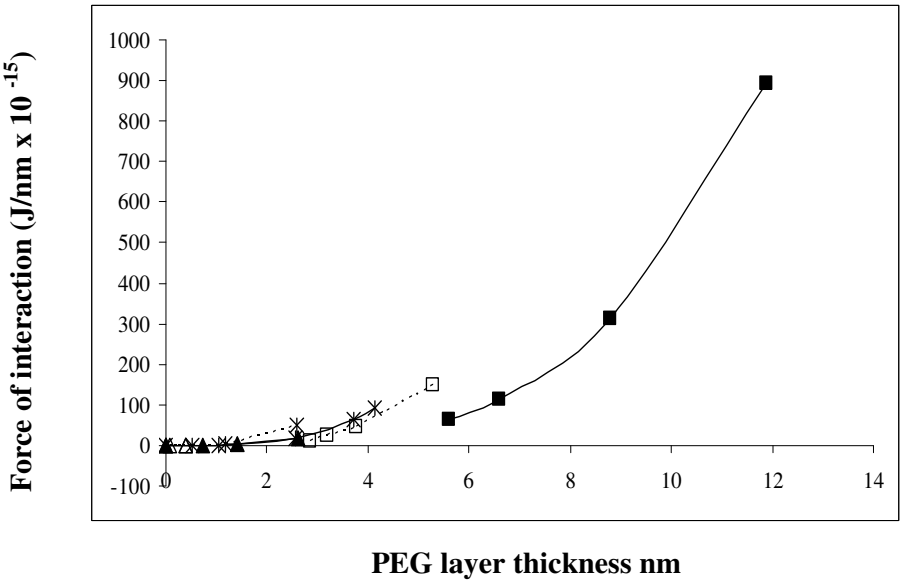


Figure 5-14: Force of interaction between protein and PEG grafted matrices (—■—PS4000, - - -□- - -PS2000, —✳—CPSP4000, - - -✳- - - CPSP2000, —▲—CMSP4000 and · · -△- · · -CMSP2000)

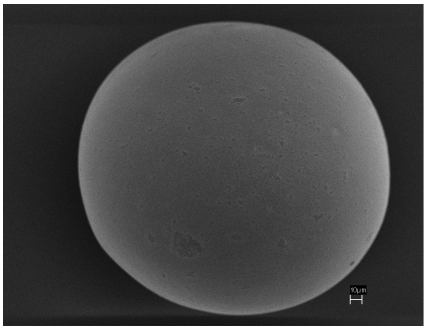
Comparing these results, the magnitude of interactive forces tends to increases sharply with the layer thickness and have a highest calculated value for polystyrene derivatives with PEG brush conformations at a layer thickness of 11.87 nm. The profile for PS4000 matrices clearly illustrates the change in interactive forces from a lower level associated

with the less grafted mushroom conformation to a higher repulsive densely grafted brush form, through an intermediate brush-mushroom overlap region. Sephadex derivatives showed a much lower interactive force, having least with CM Sephadex derivative associated with a lower PEG grafting density.

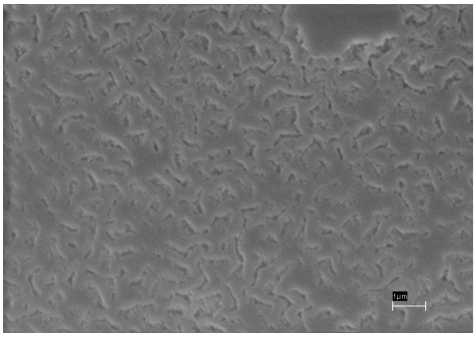
There is a possibility of discrepancies in the results due to changes in PEG layer thickness between dry and aqueous conditions, as XPS measurements were done at dry conditions and the predictions were for aqueous conditions. The values of matrix radius used in these calculations were that for the corresponding swollen matrices (Section 4.6.3.3), therefore, the only variable in these calculations is the difference in PEG layer thickness between the two conditions. However, from Equation 5.10 it is very clear that the interactive force is directly proportional to the PEG layer thickness ($F \propto t^2$), hence the magnitude on these force of interactions tends to increase substantially with increase in layer thickness but to follow the same pattern with all matrices, because all other parameters should remain the same. Consequently, these results provided a useful tool for predicting and interpreting the interaction between protein and various PEG grafted matrices.

5.4.2 Surface morphology by SEM

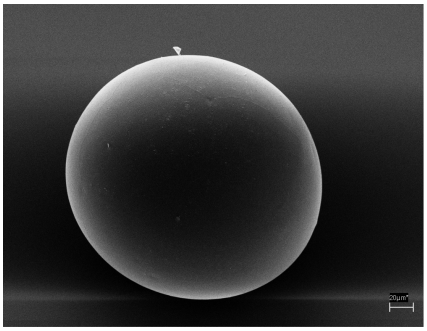
Morphological changes of the matrix surface before and after PEG grafting were observed by SEM at 5000x and 20000x magnifications. Even though it is obvious that the surface morphology of the dry and wet samples may vary substantially, the analytical constraints with the SEM technique allows the characterization of dry samples only. Surface morphologies of Sephadex, its derivatives and polystyrene beads before PEG grafting are given in Figure 5-15.



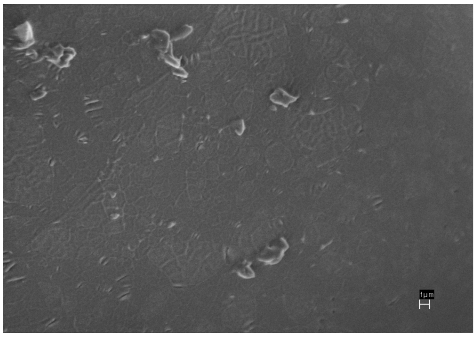
a) Sephadex G25 bead



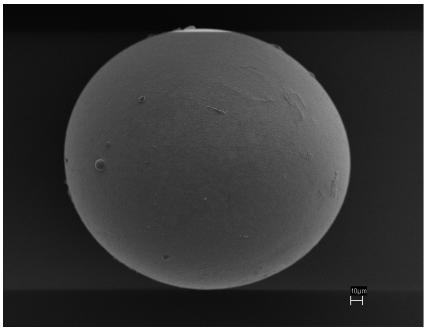
b) Sephadex G25 surface



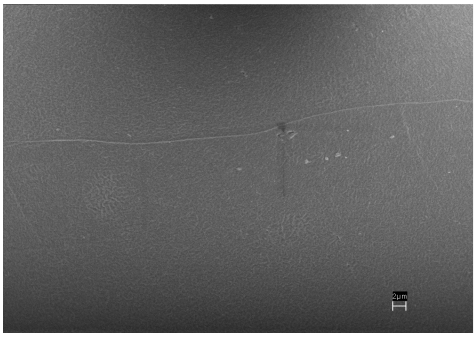
c) CM Sephadex bead



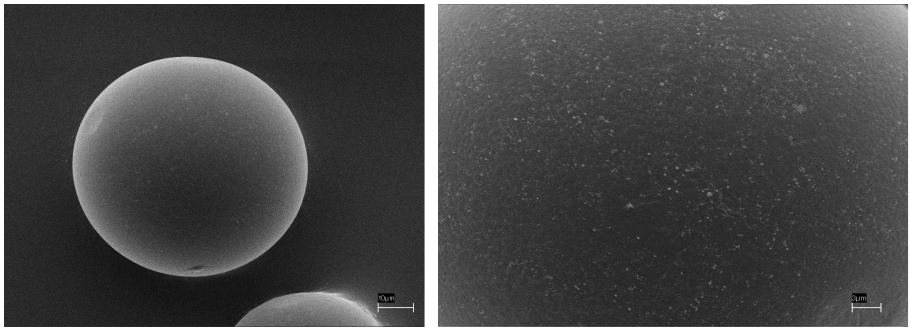
d) CM Sephadex surface



e) CP Sephadex bead



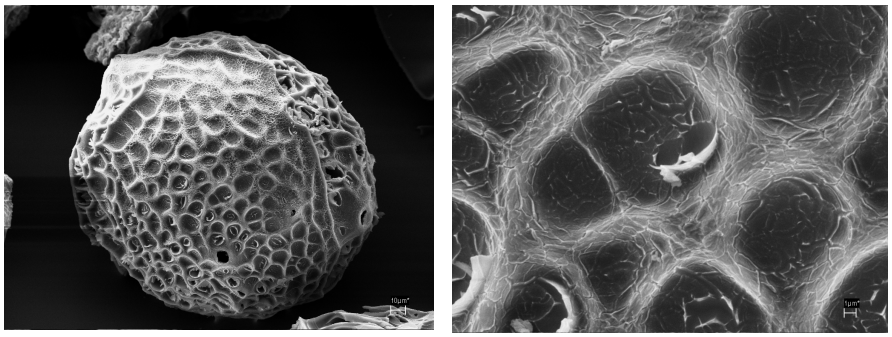
f) CP Sephadex surface



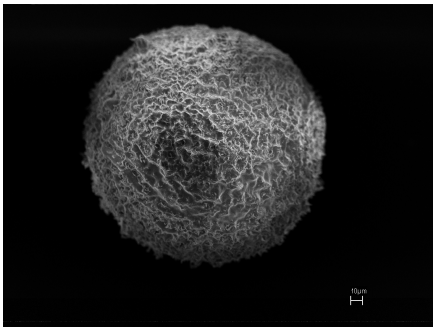
g) PS COOH bead h) PS COOH Surface

Figure 5-15: Surface morphology of Sephadex, its derivatives and polystyrene before PEG grafting (scale bars for a, c, e and g 10 µm; for b, d, f and h 1 µm)

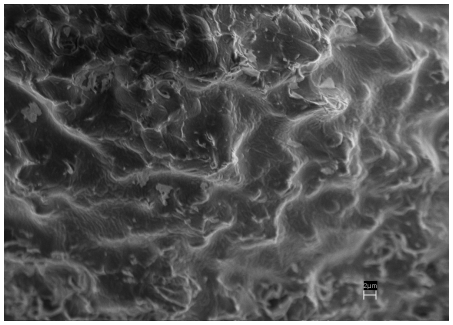
Comparing Figures 5-15a to 5-15f, a significant difference in the nature of surface characteristics between Sephadex G-25 beads and their carboxy modified derivatives was noted. The surface structure of unmodified Sephadex G 25 beads (Figure 5-15a) shows very distinctive regular convolutions, supporting the similar observation by Kocon et al. (1977). However, the introduction of carboxy functional groups into the Sephadex matrix caused considerable changes with a smoothening of the surface.



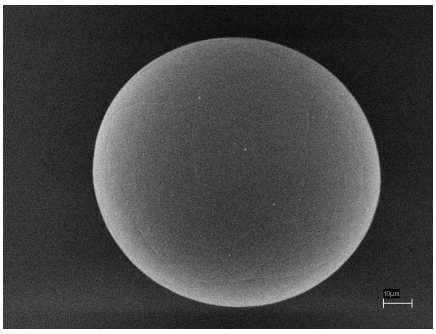
a) CM Sp-PEG bead b) CM Sp-PEG surface



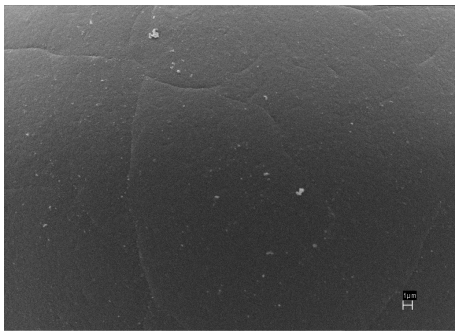
c) CP Sp-PEG bead



d) CP Sp-PEG surface



e) PS-PEG bead



f) PS-PEG surface

Figure 5-16: Surface morphology of PEG grafted Sephadex and polystyrene derivatives (PEG MW 4000, 40°C/72 hr) (scale bars for a, c and e 10 µm; for b, d and f 1 µm)

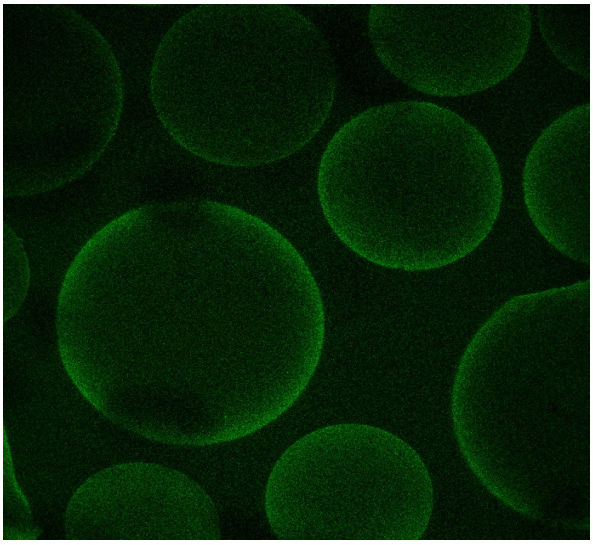
Figure 5-16 shows the surface morphologies of PEG grafted matrices, confirming the presence of PEG as a dense surface skin layer on all the samples. As seen from these figures, the surface of PEG grafted Sephadex derivatives were characterised with the formation of furrows and crests, while uniform thick coatings were observed in the case of polystyrene PEG derivatives. Formation of the distinctive surface layers of PEG on Sephadex and polystyrene matrices may arise from differences in their swelling nature in aqueous and non aqueous media. During PEG grafting, in the presence of non-aqueous media (DMF) Sephadex derivatives remained in its minimal size due to its inability to swell, while during washings it swells to its maximum size followed by shrinking during its subsequent freeze drying.

Swelling properties of polystyrene derivatives were in fact quite opposite to that of the Sephadex derivative and were in their swollen form during PEG grafting reactions, allowing maximum grafting of PEG molecules. While during its aqueous washings and freeze drying, the material shrinks to their initial size, resulting in a compact packing of the densely grafted PEG molecules and forming a uniform smooth surface throughout the matrix.

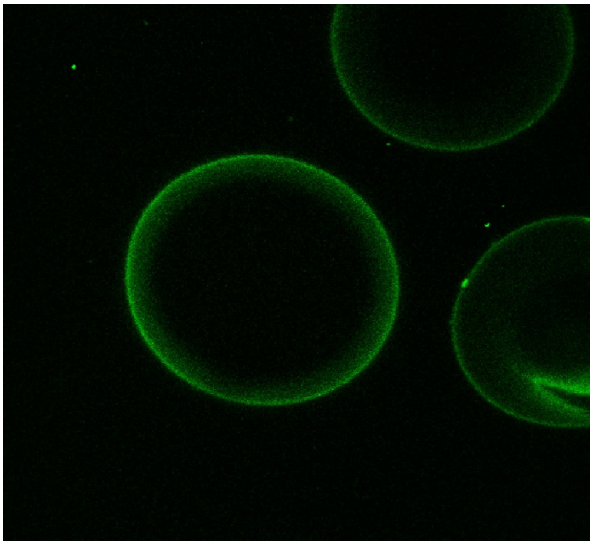
5.4.3 Fluorescent imaging by CLSM

Fluorescent images obtained from the FITC labelled Cytochrome c conjugated solid matrices were recorded by confocal laser scanning microscopy. Very distinctive fluorescent images were obtained with Sephadex derivatives confirming the presence of conjugated protein but the nature of their distribution was varied with the matrix.

Images obtained from FITC labelled Cytochrome c conjugated with the PEG grafted CM Sephadex derivative (Figure 5-17a) showed a fluorescent distribution throughout the matrix indicating an overall distribution of conjugated protein all over the matrix external and internal surfaces. In contrast, in the case of CP Sephadex PEG derivative (Figure 5-17b), fluorescence intensity was concentrated on their spherical surface which indicates a more localised conjugation of protein on their surface. Also the 3D stacking of the images (data not shown) clearly confirms the distribution of fluorescence throughout the matrix with the CM Sephadex PEG derivative but a more surface localization with the CP derivative.



a) CMSP4000–Cytochrome c FITC



b) CPSP4000–Cytochrome c FITC

Figure 5-17: Confocal fluorescent micrographs of FITC-Cytochrome c conjugated PEG-solid matrices

One of the probable reasons for this can be attributed due to the higher degree of carboxymethylation with the CM Sephadex (6.30 ± 0.36 milliequivalent/g) compared with that of CP derivative (0.98 ± 0.07 milliequivalent/g) (Section 4.6.1) and with differences in their PEG grafting densities. Lower grafting densities with higher carboxyl contents

associated with CM Sephadex derivative resulted in a substantially higher amount of unreacted carboxyl terminals on the matrix. Steric repulsive forces exerted by the grafted PEG chains resulted in an insufficient blocking of these terminals and thus generated many unblocked carboxyl group on the matrix. During subsequent NHS activation of the PEG terminal these unblocked carboxyl groups were also get activated and then conjugated with FITC labelled protein. In the case of CP Sephadex derivative, lower carboxyl content with a relatively higher PEG grafting density and subsequent ethanolamine blocking resulted in a significant reduction of unreacted carboxyl groups on the matrix. However with PEG grafted polystyrene derivatives, conjugation with Cytochrome c was not successful even after prolonged conjugation time and hence resulted in no colouration and no fluorescence in the resultant matrix.

These observations showed a very significant correlation with the predicted results using Equation 5-10, illustrated in Figure 5-14. CM Sephadex-PEG derivatives, which showed the lowest interaction energy among the PEG grafted matrices, allows a substantial amount of proteins conjugated on the matrix surfaces, arising from primary adsorption. Consequently, there is an overall fluorescent distribution observed along the matrix surface. With an increase in repulsive interactions, a comparatively lower fluorescent intensity was observed with CP Sephadex derivatives, resulting from less protein conjugation. The repulsive interactions showed the highest values with polystyrene matrices, which prevented protein conjugation, even on the secondary potential energy minima region near the PEG terminals, and hence no fluorescent emission was observed with this matrix.

5.5 Conclusions

Surface characterization and quantification of various PEG grafted solid matrices contributed towards much valuable information regarding their surface concentration, morphology, conformational properties and the nature of interaction between the matrix and approaching protein during conjugation. Correlating the C-O peak intensities obtained from a high resolution C 1s XPS scan provided a useful tool for quantifying surface grafted PEG molecules. Quantification, followed by measuring grafting densities and distance between grafted molecules, yielded a very useful methodology to distinguish the various conformational forms of surface grafted PEG molecules.

A layer thickness of 11.87 nm, calculated for PEG grafted polystyrene matrices with a brush conformation, is comparable with that of other reported results, indicating the validity of these results. Simultaneous determination of layer thickness as well as the distance between the polymer chains using XPS provided a superior methodology for conformational assignment of the grafted PEG molecules than other reported methods. XPS results also showed that the nature of the solid matrix has a great impact on the grafting kinetics and conformational properties, with polystyrene matrices resulting in PEG brush conformations at a high grafting density, whereas Sephadex derivatives resulted in only mushroom conformations, with lower grafting densities. Also, the influence of tether length and steric hindrance near the conjugation sites were demonstrated by the XPS results, showing CP Sephadex (with a comparatively longer carboxyl terminus) yielded a higher PEG grafting density than that of the CM Sephadex derivative with only a single carbon tether length. Correlation of PEG layer thickness with energy of interaction between protein and PEG grafted

matrix confirmed the existence of a higher energy barrier for the approaching protein and polystyrene derivative with brush conformations compared to other PEG grafted matrices and conformations.

Surface morphological information provided by SEM shows distinctive differences between various PEG grafted matrices due to their differences in grafting densities. Fluorescent imaging by confocal microscopy provided a visual conformation for the protein conjugation on the solid-phase PEGylation matrix.

6 Constraints behind solid-phase protein PEGylation – a comprehensive discussion

6.1 Introduction

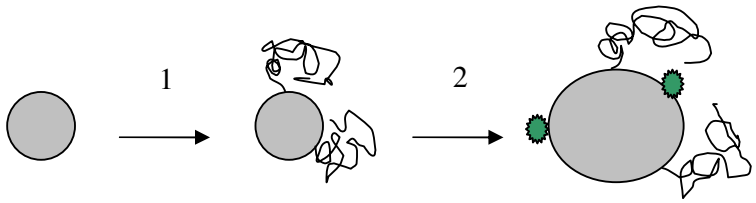
This chapter is aimed at a detailed discussion of the current results of the solid-phase protein PEGylation system and various molecular mechanisms and forces underlying the constraints and bottlenecks of the process. Solution-phase PEGylation using the same PEG derivatives was performed satisfactorily, as expected from the reported literature, whereas the same PEG derivatives resulted in a very different product profile and performed in an entirely different way when grafted onto a solid matrix. Quantification and conformational studies of grafted PEG molecules using XPS provided very useful understanding and insights into the various mechanisms and reasons for all these observations and results.

6.2 Solid-phase protein PEGylation – proof of concept

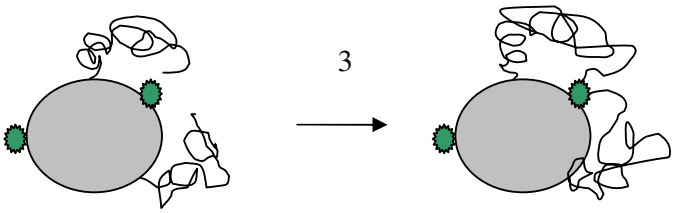
The presence of PEGylated Cytochrome c derivatives in the hydrolysed fractions obtained from both CM and CP Sephadex derivatives characterised by SEC analysis (Figure 4.18 to 4.20) provided a proof for the concept. Even though the process was expected to deliver predominantly mono-PEGylated species using tethered PEG molecules, by limiting the mobility of the polymer, only multi-PEGylated species were present in the hydrolysed fractions. SEC results of solution-phase PEGylation using the same PEG derivatives (Figure 3-14 and 3-15) also resulted in a mixture of PEGylated products but they were characterised by a substantial amount of mono-PEGylated species in the product mixture. Comparing these two set of results; it is quite obvious that the

product obtained from the solid-phase system comprises a variety of multi-PEGylated product with very little or almost nil mono-PEGylated species.

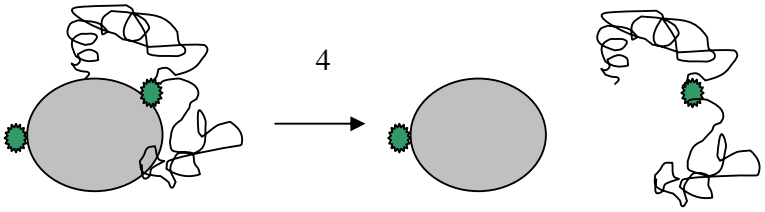
The formation of a multi-PEGylated product from PEG derivatives of two different matrices is very unlikely, due to the continuous uniform movement of the reaction mass during protein conjugation. Therefore the source of PEG molecules for this multiple conjugation on a single protein should be from a single matrix with close proximity to the protein. This suggests the possibility of Cytochrome c attached to surface carboxyl groups of the matrix through ion exchange interactions resulting from primary adsorptions, which were then conjugated with the NHS activated PEG molecules present nearby, resulting in multiple conjugated products. The presence of an ion exchange interaction between the matrix and the protein is obvious, because if the protein was conjugated through an amide bonding through NHS activation, its release under the mild hydrolysis conditions was not possible due to the stability of amide linkages. Based on these findings, the following mechanism can be formulated for this result, as illustrated in Figure 6-1.



Step 1 and 2 Swelling of Sephadex matrix during protein conjugation in aqueous solution and ion exchange interaction between the positively charged protein N-terminal and negatively charged unmodified carboxyl group on the surface



Step 3 Conjugation of near by NHS activated PEG molecules with protein



Step 4 Hydrolysis and release of multi-PEGylated species
Figure 6-1: Mechanism for formation of multi-PEGylated products

The swelling nature of Sephadex derivatives is well known and a size comparison using the optical microscopic studies (Section 4.6.3.3) showed the sizes of particle were increased between 1.5 to 1.8 times their initial sizes when swelled in aqueous medium. This swelling of the porous Sephadex derivatives can be broadly divided into two steps, first an instantaneous filling of the cavities of the porous matrix by capillary force, followed by the osmotic diffusion of aqueous solution into the matrix (Pratt and Coony 1973; Ikkai and Shibayama 1996). This process is accompanied by the ionisation of a number of unblocked carboxylic groups, which were hidden underneath the matrix surface and were unavailable for NHS activation and for the subsequent ethanolamine blocking, resulting in an increase in hydrophilicity of the matrix (Zagorodni 2007). Consequently, a net attractive force was exerted on the approaching protein towards the matrix surface. This was further

favoured by the presence of lower PEG grafting densities, confirmed from their gain in weights (Figure 4-12 to 4-14) and XPS studies (Figure 5-8 to 5-10), and provides sufficient space for the conjugation of Cytochrome c on some of their unmodified surface carboxylic groups without much repulsive interaction. The presence of traces of Cytochrome c with some SEC results, even in the absence of any PEGylated species, confirm these ion exchange interactions and thus support this hypothesis for the formation of multi-PEGylated products.

A reduced number of repulsive interface interactions, exerted by PEG grafted Sephadex derivatives towards the approaching protein, were anticipated, due to their inherent swelling property and porosity (Section 2.6.2.1). However the reduced grafting density and resulting random coil mushroom conformation of PEG molecules inversely affected the process, mainly resulted in primary adsorptions of proteins on the matrix surface. Higher grafting concentrations were not achieved due to increased repulsive interactions between PEG molecules during grafting and also due to the small surface area of the matrix under non-aqueous conditions and the resulted crowding near the reaction sites. A better product profile could have been achieved with these Sephadex derivatives if they had a high grafting density and then the interchain repulsions between the hydrated chains (Kingshott et al. 2002) would enable the polymer chains to keep them apart to facilitate a predominantly single chain conjugation on the approaching protein (Figure 6-2).

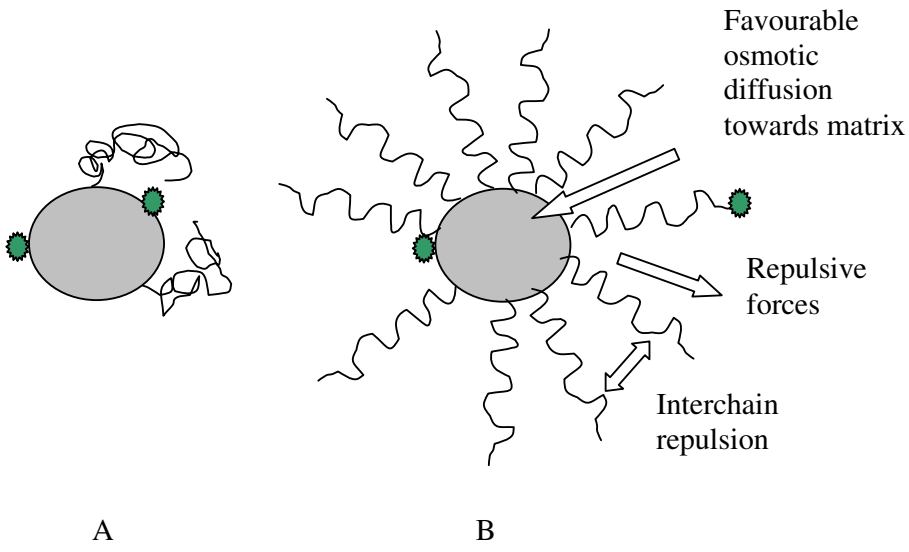


Figure 6-2: A) PEG low grafting density and mushroom conformation and B) PEG high grafting density and brush conformation using Sephadex derivatives (proposed)

6.3 Solid-phase protein PEGylation – constraints and bottlenecks

Overall, from the various positive and negative results obtained, it is clearly understood that cumulative surface force interactions contributed by various thermodynamic and kinetic controlled factors predominate over the specificity of chemical conjugation. A localised protein concentration near the NHS activated PEG terminals were anticipated as a result of the net outcome of the repulsive and attractive interactions (Figure 2-26) and thereby expected selective mono-PEGylation through NHS activated PEG terminals, which were separated from each others due to interchain repulsions. The following factors were found to influence the outcome of the process to a great extent.

6.3.1 PEG grafting density

Very low PEG grafting densities were obtained with both Sephadex derivatives, which resulted in predominantly primary adsorption of Cytochrome c on the surface matrix. The use of five carbon spacer with an improved grafting profile also did not provide satisfactory results. The difference in the swelling nature of the Sephadex matrix between the solvents during PEG grafting and protein conjugation resulted in several unblocked carboxyl functional groups becoming available for nonspecific protein interactions.

6.3.2 Surface force interactions and nature of the matrix

Surface force interactions as well as the nature of the matrix played key roles in this process. The directions of the cumulative force of the interactions were entirely different with Sephadex and polystyrene matrices. In the case of Sephadex derivatives, with very high porosity and swelling behaviour in aqueous medium, there was an overall attractive osmotic diffusion towards the matrix during swelling (Pratt and Coony 1973), which resulted mainly in the primary adsorption of protein on the matrix surface due to lower PEG grafting density and mushroom conformations. Sephadex derivatives were reported with a net inner osmotic pressure of the order of 10^5 Pa (Ogston and Wells 1972), confirming the possibility of a very high positive solvent intake towards the matrix while swelling.

In the case of polystyrene, the hydration of grafted PEG molecules resulted in the formation of a highly concentrated polymer solution surrounding the matrix. This resulted in an increased repulsive force towards the approaching protein according to Equation 2-1, caused

mainly by the increased osmotic pressure. Unlike in the case of Sephadex matrices, where the highly swollen and porous matrix facilitated an inward osmotic diffusion towards the matrix, the non-swelling behaviour of polystyrene in aqueous solution resulted in collective repulsive interactions away from the matrix surface. An increase in PEG surface concentrations contributed a proportional increase in the concentration of this virtual polymer solution and reached a maximum value with the PEG brush conformation. This resulted in the maximum possible repulsive interaction during protein conjugation, thereby preventing protein from being directed towards the NHS-activated PEG terminal.

The magnitude of repulsive interactions between the approaching protein and various PEG grafted matrices, illustrated in Figure 5-14, clearly indicates the existence of a very high interaction energy with polystyrene matrices with PEGs in their brush conformations. Due to lack of dissipation of this high energy through the matrix surface, proteins were repelled farther away even from the PEG terminals; thereby preventing conjugation through the NHS-activated terminals. Furthermore, these predictions showed that the repulsive interactions were a function of PEG grafting density and molecular weights of PEG used and increased with increasing surface concentration and molecular weight. Consequently, overall protein repulsion was associated with densely grafted PEG polystyrene matrices, with PEGs in their brush conformations that prevented protein from conjugation to the NHS-activated PEG terminals.

6.3.3 Parallel hydrolysis of NHS ester

Although NHS ester is used extensively for protein conjugation due to its selectivity and specificity towards N-terminal amine coupling, the half-life period of NHS ester under aqueous conditions is between 20 and 30

minutes only (Roberts et al. 2002). This contributed another important constraint for conjugation in the presence of repulsive surface interactions especially with the polystyrene derivatives.

6.3.4 Yield of hydrolysis

The present yield of hydrolysis using 0.2 M glycine-NaOH buffer (pH 8.5) at 20°C was only about 12% of the grafted PEG, suggesting a very low rate of efficiency of the hydrolysis (Figure 4-17). Because the primary objective was to prove the chemistry of this novel technology, the yield of hydrolysed product was only of secondary concern and it might be hoped that yield could be improved through further research. However, the use of an alternative, stronger hydrolysing agent and conditions to achieve a better yield were restricted, due to the presence of protein in the reaction media and the high possibility of denaturation in the presence of strong chemicals and reaction conditions.

6.4 Conclusions

From a detailed analysis of the various results and a number of contributing factors, it can be concluded that the results were greatly influenced by thermodynamic and kinetic factors, rather than the specificity and selectivity of the chemistry involved. The structural properties of the polymer chain, as well as nature of the matrix, contributed adversely towards the overall outcome of the process. The protein repulsion behaviour of grafted PEG chains predominates, rather than the anticipated selective conjugation behaviour of the tethered molecule.

7 Conclusions and recommendations

7.1 Conclusions

The requirement of an improved PEGylation technology to meet its growing pharmaceutical importance is a significant issue in modern drug delivery research. To contribute towards this important field of research, the development of a novel solid-phase protein PEGylation was attempted with a major emphasis on reducing the product heterogeneity and improving the yield. The overall outcomes of the research are summarised in the following sub-topics:

7.1.1 Synthesis of heterobifunctional PEG derivatives

A range of novel heterobifunctional PEG derivatives were prepared and characterized in the laboratory. PEG derivatives functionalized with the cleavable β-alanine terminal can be used for a wide variety of applications, including releasable drug delivery and solid-phase synthesis, due to their characteristic kinetic labile nature. A solution-phase protein PEGylation using PEG derivatives prepared in this work resulted in comparable product profiles to those obtained using commercially available activated PEGs.

7.1.2 PEG surface grafting and characterization

Optimized PEG grafting conditions were established using non-aqueous amine conjugation chemistry and the influence of various parameters and the nature of the solid matrix on PEG grafting were studied. The characterization of PEG grafted matrices using XPS resulted in the

drafting of a useful methodology to differentiate the various conformational behaviours of grafted PEGs. The approach to distinguishing between PEG conformations using XPS, described in this thesis, has potential as a useful tool in characterising and studying the conformational behaviour of various PEG grafted matrices for a wide variety of applications.

7.1.3 Performance of the solid-phase PEGylation system

Using the solid-phase PEGylation resin developed in this work, conjugation with the model protein Cytochrome c was attempted. Even though a predominant mono-PEGylation, as anticipated based on the theoretical predictions, was not achieved, experiments with Sephadex derivatives resulted in multi-PEGylated products, which to our knowledge is the first attempt at protein conjugation using a convalently attached, solid-phase PEGylation resin.

7.1.4 Constraints behind the solid-phase PEGylation system

The overall performance of this system is a cumulative outcome of a number of factors operating simultaneously, including various surface force interactions, PEG grafting density, the nature of the solid matrix, the half-life of the PEG terminal functional group, the latter's availability near the suitable protein functional group for conjugation and finally the yield of hydrolysis of PEGylated protein from the matrix. Summarizing the detailed interpretation of these results and various contributing factors given in Chapter 6, it can be concluded that surface force interactions dominated over conjugation chemistry and contributed adversely to the process. A systematic and detailed study of various parameters imparted

a very good understanding of various constraints and provided a useful scientific background for future developments.

7.2 Recommendations

The following recommendations are identified for further improvement to the solid-phase PEGylation technique:

- The use of PEG derivatives activated with a stable functional group other than NHS group to withstand the parallel hydrolysis during protein conjugation. Such a long stable functional group may be useful particularly with polystyrene derivatives, where the initial high osmotic pressure when immersed in the aqueous solution may subside over a period of time and thereby protein can reach the PEG terminal.
- The use of selectively and quantitatively cleavable protein tags (Section 2.6.3) to increase the yield of hydrolysed PEGylated product without denaturing the protein.
- The use of a porous matrix capable of providing a very good PEG loading (compared with polystyrene) but capable of reducing the osmotic pressure around the interface, through an inward osmotic diffusion as in the case of Sephadex derivatives. The use of such matrices enables the formation of PEG brushes on the matrix surfaces, and the inward osmotic diffusion towards the matrix surface enables protein accumulation on the secondary potential energy minima near the PEG terminals and thereby facilitates protein conjugations.
- This concept can be successfully extended for PEGylating synthetic drugs and similar applications. In this case, parallel hydrolysis of the NHS group can be avoided by conducting non-

aqueous conjugations or directly applying a catalyst mediated covalent coupling between the terminal PEG functional group and a suitable functional analogue available with the drug. Furthermore, quantitative cleavage of the product can be achieved with the help of a suitable reagent without affecting the PEGylated product.

8 References

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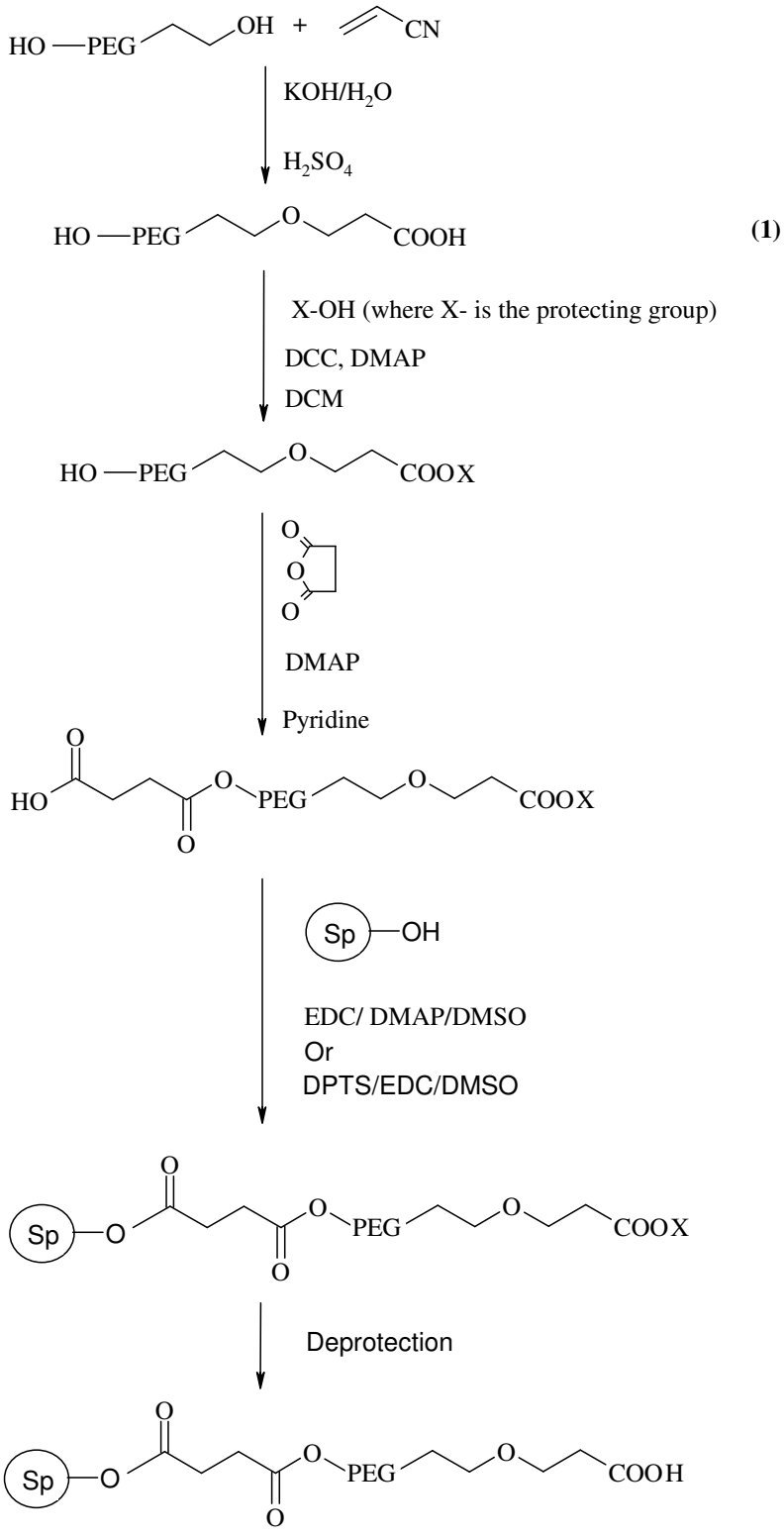
9 Appendix-A: Use of succinyl ester as the cleavable linker for solid-phase protein PEGylation

A.1 Introduction

Initial methodology for preparing a solid-phase protein PEGylation system was proposed based on using a succinyl ester as the cleavable linker between the functionalized PEG and the solid matrix. The heterobifunctional PEG derivative for this modification was prepared from its monocarboxy derivative as mentioned in Chapter 3. The carboxy terminal of the PEG derivative was first protected and the unmodified hydroxyl terminal was then converted into its succinyl ester derivative and finally grafted onto the solid matrix. The concept behind this was to conjugate protein through deprotected carboxy terminal of the PEG derivative after activating with its NHS ester and finally cleaving off from the resin through the hydrolysis of the labile succinyl ester linkage.

A.2 Synthetic scheme

The overall reaction scheme used for the process is summarized in Figure A-1. α-hydroxy-ω-carboxy PEG **(1)** was prepared by following the same method given in Scheme-1 and Scheme-2 (Figure 3-4 and 3-5) by using acrylonitrile and conc. H_2SO_4.


HO —PEG OH + CN
KOH/H2O
H2SO4
HO —PEG O COOH
(1)
X-OH (where X- is the protecting group)
DCC, DMAP
DCM
HO —PEG O COOX
DMAP
Pyridine
HO O PEG O COOX
Sp —OH
EDC/ DMAP/DMSO
Or
DPTS/EDC/DMSO
Sp O PEG O COOX
Deprotection
Sp O PEG O COOH

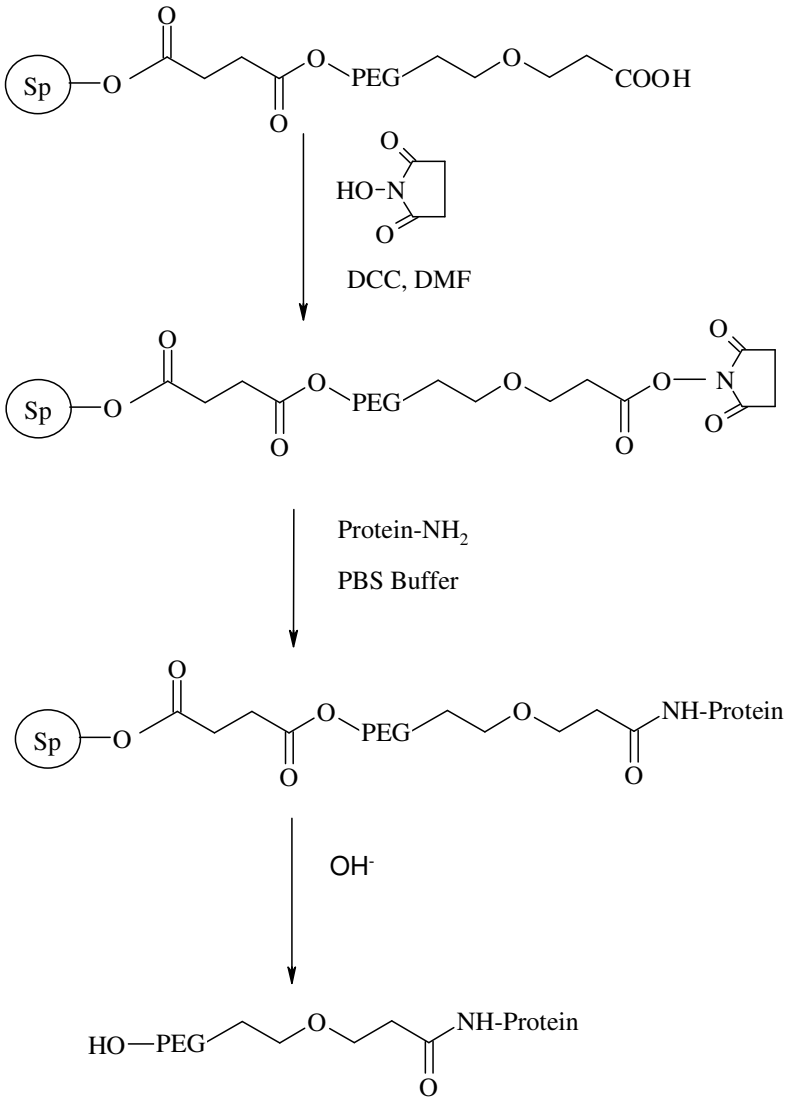


Figure A-1: Synthetic scheme for the solid-phase protein PEGylation using succinyl ester linkage

A. 3 PEG grafting

Because the postulated synthetic scheme utilizes a solid-phase deprotection of the grafted PEG, carboxyl protecting groups were selected accordingly which can be easily removed under heterogeneous reaction conditions. In one strategy the carboxyl protection was performed by converting into its p-methoxy-α-methyl benzyl ester due to its selectivity towards the oxidative cleavage under mild conditions using 2,3-dichloro-5,6-dicyano benzoquinone (DDQ)(Yoo et al. 1990; Wu et al. 2001) or by using ceric ammonium nitrate (CAN) (Johansson and Samuelsson 1984; Jarowicki and Kocienski 2001). p-methoxy-α-methyl benzyl PEG ester was prepared by using DCC and DMAP in anhydrous DCM, following the same conditions as referred in Section 3.5.1.2. ^{1}H NMR ($CDCl_3$): PEG back bone (δ 3.6292 ppm), -CH_2-C**H**$_2$-COO- (t, δ 2.629 ppm), -COO-C**H**-(CH_3)-benzyl (q, δ 5.845 ppm), -COO-CH-(C**H**$_3$)-benzyl (d, δ 1.496 ppm), benzyl Ar **H**s (dd, δ 6.845 and 7.258 ppm) and benzyl-O-C**H**$_3$ (s, δ 3.782 ppm).

Solution-phase deprotection of the PEG-benzyl ester using both DDQ (2.5 molar eq. in DCM/H_2O) and CAN (2.8 molar eq. in acetonitrile/ H_2O) were resulted in only the partial removal (60 to 70%) of the benzyl ester group (monitored by ^{1}H NMR). Simultaneous reduction of the oxidizing agents during the deprotection imparted dark reddish brown colouration to the PEG derivative in both cases. The reduced quinol product of DDQ crystallises along with the PEG derivative from the diethyl ether due to its insolubility in nonpolar solvents (Lee-Ruff and Ablenas 1989) and hence it was difficult to remove from the products during the solution-phase reaction monitoring studies. Also the solid-phase oxidative deprotection of the grafted PEG with these reagents resulted in intensive colouration to the resin, even after extensive

washing with aqueous sodium chloride solution and water. Lack of a complete deprotection even with a homogenous solution-phase reaction and the inability for complete removal of reaction by-products with a heterogeneous reaction system insists the requirement for an alternative deprotection method.

Merits of allyl protection and its deprotection using soluble palladium catalysts were described in Section 3.2.1.6. A complete removal of allyl protecting group was achieved with $Pd(PPh_3)_4$ in presence of various nucleophilic allyl scavengers such as phenylsilane, NMM and pyrrolidine (Section 3.5.1.4).

After protecting the ω-carboxyl terminal by converting into its allyl ester (Section 3.5.1.2), succinyl ester moiety was introduced on the free hydroxy terminal of the PEG derivative. Succinyl ester of the allyl protected PEG derivative was prepared by reacting with succinic anhydride in anhydrous pyridine in presence of DMAP (Douglas et al. 1991; Krepinsky et al. 1994). ^{1}H NMR ($CDCl_3$) (Figure A-2): Succinyl –CH_2-CH_2- (m, δ 2.573 ppm), -CO_2-CH_2-CH_2-O- (t, δ 4.191 ppm), PEG back bone (δ 3.587 ppm), -O-CH_2-CH=CH_2 (d, δ 4.534, 4.545 ppm), -O-CH_2-CH=CH_2 (m, δ 5.849 ppm), -O-CH_2-CH=CH_2 (dd, δ 5.162, 5.183 and 5.242, 5.279 ppm), DMAP pyridine **H**s (d, δ 6.675, 6.689 and 8.171, 8.184 ppm) and DMAP –N(CH_3)$_2$ (s, δ 3.173). (NMR spectrum indicates that the product exist as DMAP salt of the succinic acid derivative.)

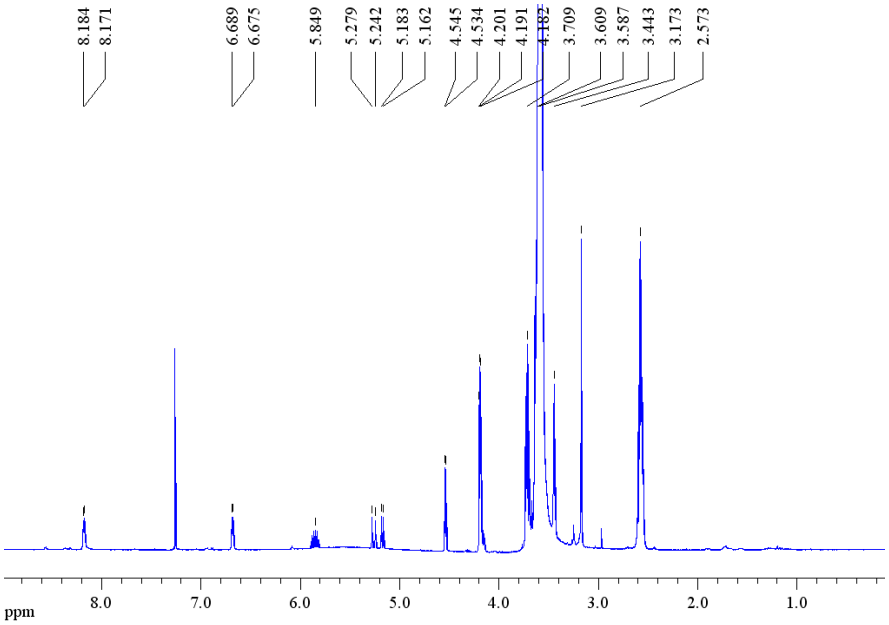


Figure A-2: ^{1}H NMR of α-succinyl-ω-allyl PEG derivative

Grafting of this succinyl PEG derivative onto Sephadex was attempted by various methods. In all experiments 25 molar eq. of PEG derivative per g dried Sephadex were reacted and after completion of the reaction products were washed extensively and successively with DI water, 2 M NaCl and DI water and freeze dried for 48 hour, till a constant weight is achieved. Experiments were performed in triplicates and the averages values were taken for yield calculations. The gain in weight was directly correlated to the amount of PEG grafted and finally expressed in terms of its molar equivalents. The results are summarized in Table A-1.

Table A-1: Summary of PEG grafting through succinyl ester linkage

Reaction conditions	Reference	PEG µmol/g modified Sephadex
DMF / Toluene (1:2), 100°C / 12 hr.	(Huang et al. 2006)	1.10 ± 0.19
Sephadex modified with p-toluene sulfonyl chloride (TsCl) (1.6g TsCl / g dry Sephadex), followed by reaction in anhydrous pyridine, 25°C/72 hr.	(Köster and Heyns 1972; Dharmaratne et al. 1982)	1.31 ± 0.28
p-Toluene sulfonic acid (TsOH, 0.1 eq$^{\#}$) / EDC(1.5 eq$^{\#}$) / DMSO, 25°C / 72 hr.	(Holmberg and Hansen 1979)	1.56 ± 0.25
DPTS* (0.15 eq$^{\#}$) / EDC (1.5 eq$^{\#}$) / DMSO, 25°C / 72 hr.	(Hiemstra et al. 2007)	24.81 ± 4.51

Notes: *Based on PEG derivative molar ratio.
*DPTS: 4-(N,N-dimethylamino) pyridinium-4-toluene sulfonate, prepared from TsOH and DMAP in anhydrous THF (Zubarev et al. 2006).

Comparing the above results, a better PEG grafting density was observed when DPTS was used as the catalyst. FTIR spectra of Sephadex and PEG grafted Sephadex (using DPTS) were given in Figure A-2. Characteristic broad peak around 2900 $cm^{-1}$and from 1000 to 1100 cm^{-1} confirming the presence of PEG on the modified Sephadex derivative (Section 4.6.3). This can be further confirmed from the diminished intensity of the –O-H stretching frequency at 3650 cm^{-1} of Sephadex derivatives due to the PEG grafting.

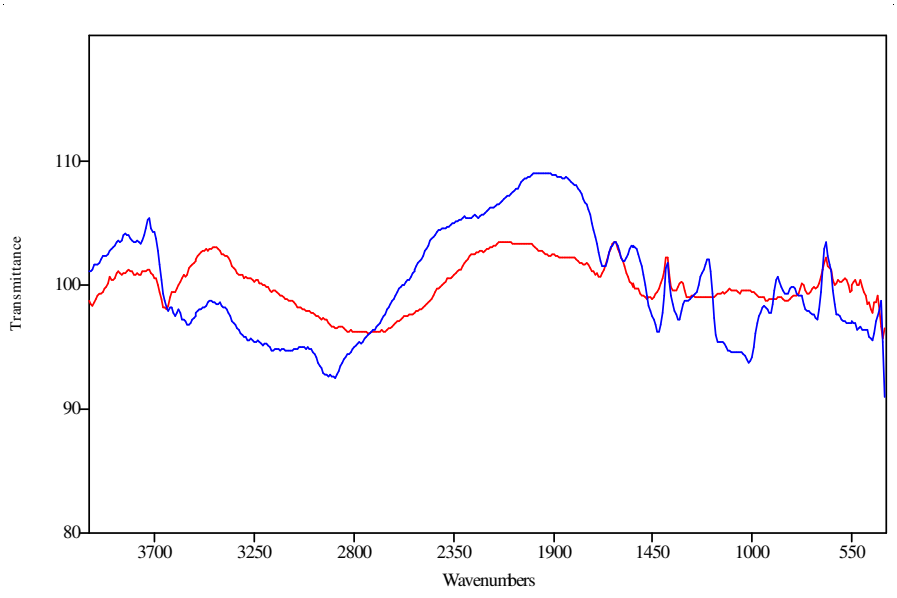


Figure A-3: FTIR of Sephadex and its PEG derivatives (——Sephadex and ——CM Sp-PEG (using DPTS)

A. 4 PEG hydrolysis from the resin

Succinyl ester linkages are characterised with its labile nature under mild conditions (Section 3.2.1.2). Use of mild and non nucleophilic bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in cleaving this linker are getting more attraction in solid-phase synthesis due to its versatility in cleaving this ester linkage almost quantitatively, under mild reaction conditions. DBU is particularly proved its capability in cleaving PEG molecules anchored through succinyl ester linkage onto various oligosaccharides without affecting any other functional groups in many solid-phase peptide syntheses (Baptistella et al. 1989; Douglas et al. 1991; Seebach et al. 1991; Krepinsky et al. 1994).

PEG grafted Sephadex derivatives through succinyl ester linkage were treated with DBU under various conditions and the amount of PEG cleaved off from the solid matrix were estimated by using the ammonium

ferrothiocyanate-chloroform method (UV estimation at 510 nm) against standard solutions of known concentrations (Nag et al. 1996). Reaction profile of PEG release from its Sephadex derivative under various conditions are summarised in Figure A-3.

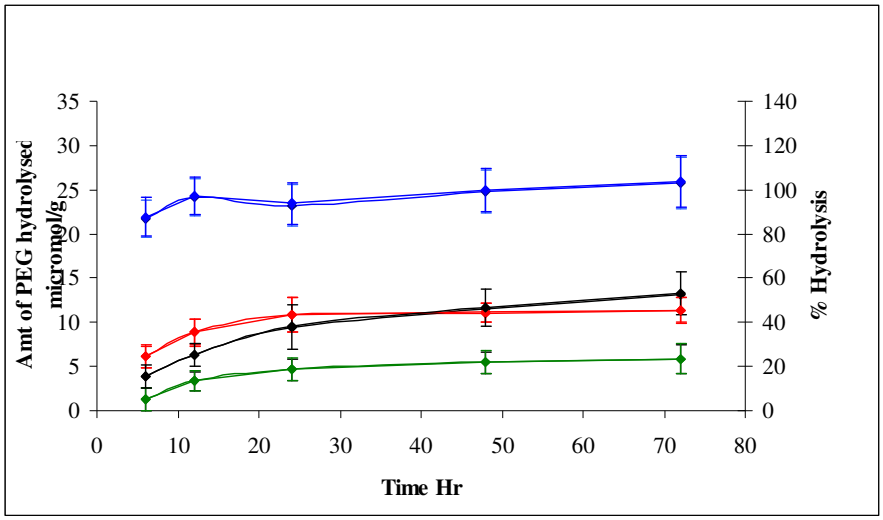


Figure A-4: Release of PEG by hydrolysis of succinyl linkage (♦- 2 eq. DBU/5 eq. LiBr/MeOH, ♦- 5 eq. DBU (in H_2O), ♦- 2 eq. DBU (in H_2O) and ♦- 0.2 M Glycine-NaOH buffer pH 8.6)

Almost quantitative release of PEG was observed when DBU/LiBr/MeOH mixture was used (Seebach et al. 1991). But this method was not adaptable for cleaving the protein conjugated PEG derivatives, since presence of lithium bromide can induce denaturing of the protein. Aqueous solutions of DBU were also resulted in hydrolysis of the succinyl linkage, but with lower yields. Use of 0.2 M Glycine-NaOH buffer resulted in a comparatively similar hydrolysis profile with these aqueous DBU solutions, with a steady increase in the yield with reaction time. With a compromise in the yield of PEG release, 0.2 M Glycine-NaOH buffer was selected for further experiments in order to prevent any protein denaturing.

A. 5 Solid-phase protein conjugation

After establishing the grafting and cleaving conditions, PEG grafted Sephadex derivative was subjected for allyl deprotection using $Pd(PPh_3)_4$ under anhydrous reaction conditions (Section 3.5.1.4). Experiments were performed with phenylsilane, NMM and pyrrolidine as the nucleophilic allyl scavengers. Due to heterogeneity of the reaction media, it was not possible to understand the reaction kinetics and extend of allyl deprotection. In absence of a suitable reaction monitoring methodology, the deprotection was repeated twice, and the resin after the deprotection was reacted with NHS in presence of DCC in anhydrous DMF for the activation of the free terminal carboxyl groups.

The NHS activated resin was then tried to conjugate with Cytochrome c (1mg/ mL) in 10 mM PBS buffer after equilibrating the resin in the same buffer. After treating the resin with protein solution for 2 hour at 20°C, the resin was filtered and washed thoroughly with 2 M NaCl solution and then with DI water. Compared the UV absorption (at 280 nm) of the filtrate after conjugation with that of the initial Cytochrome c solution and found absence of any protein conjugated onto the solid matrix. This was also confirmed from the absence of any characteristic reddish brown colouration to the solid matrix after washings, arising from the Cytochrome c conjugation. In order to reconfirm this, the resins were further treated with 0.2 M Glycine-NaOH buffer solution and fractions were analysed by SEC. But none of the fractions were detected any PEGylated species in any of these analysis.

A.6 Conclusions

Because the only available carboxy functional group is with the PEG terminal, any nonspecific protein conjugation with the solid matrix was not possible with this modified resin, which was the main advantage and characteristic feature of this reaction scheme. Also the presence of highly labile succinyl ester linkage between the PEG and the matrix provided a comparatively higher yield of PEG after hydrolysis than compared with that from the β-alanine linkage.

One of the main reasons for the failure of this scheme can be attributed to the heterogeneity of the reaction media while deallylation, which restricts the availability of a suitable methodology for reaction monitoring. Even though it was possible to monitor and understand the reaction profile in homogenous solution-phase very accurately, the extent of deallylation with the solid-phase system is still uncertain. During this development, the conformational constraints of the tethered PEG molecule were not well understood and studied due to the unavailability of its analytical tools. This important factor may also played a crucial role during the protein conjugation.

With the high mobility of the PEG chain in the aqueous media and increased osmotic pressure due to the hydrated PEG coils, there is an increased repulsive forces exerted on the approaching proteins for conjugation. Along with these repulsive forces, the conformational behaviour of the tethered PEG molecule and the very less half life values of the active NHS ester may resulted in the complete hydrolysis of the available NHS activated PEG terminals, before a protein could reach these active sites.

10 Appendix-B: Supplementary XPS results

B.1 Survey scans

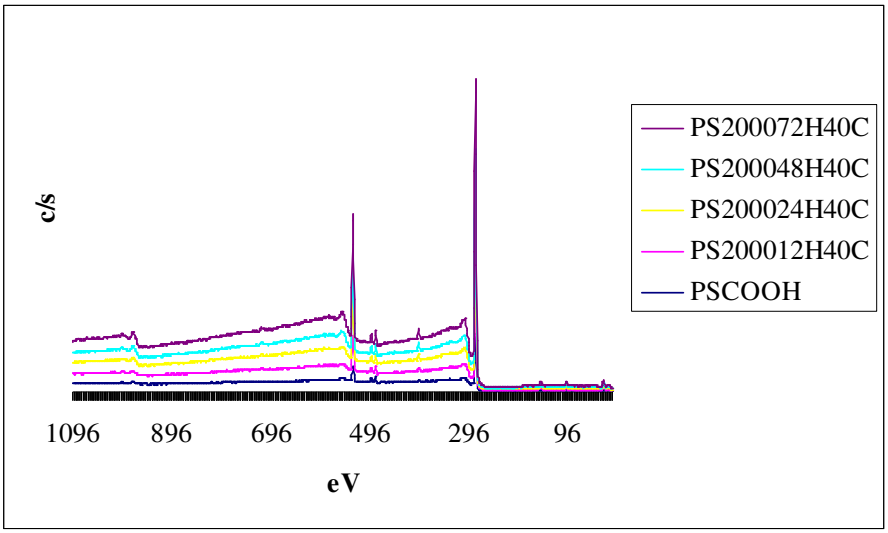


Figure B-1: Survey scans of PEG (2000 Da) grafted polystyrene

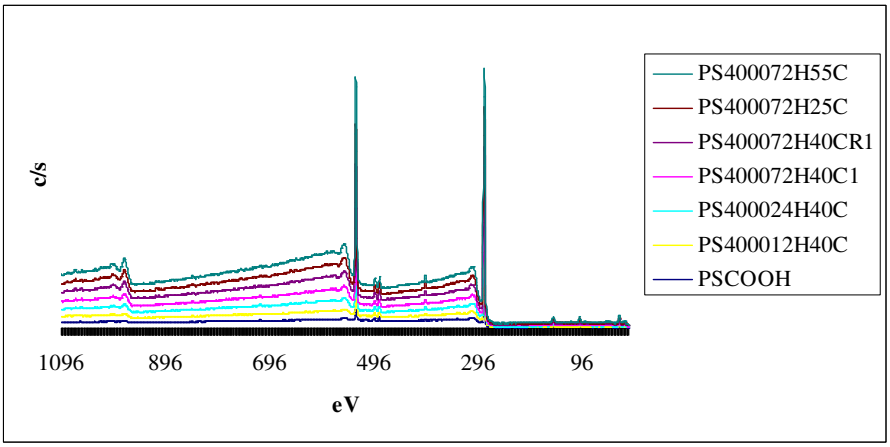


Figure B-2: Survey scans of PEG (4000 Da) grafted polystyrene

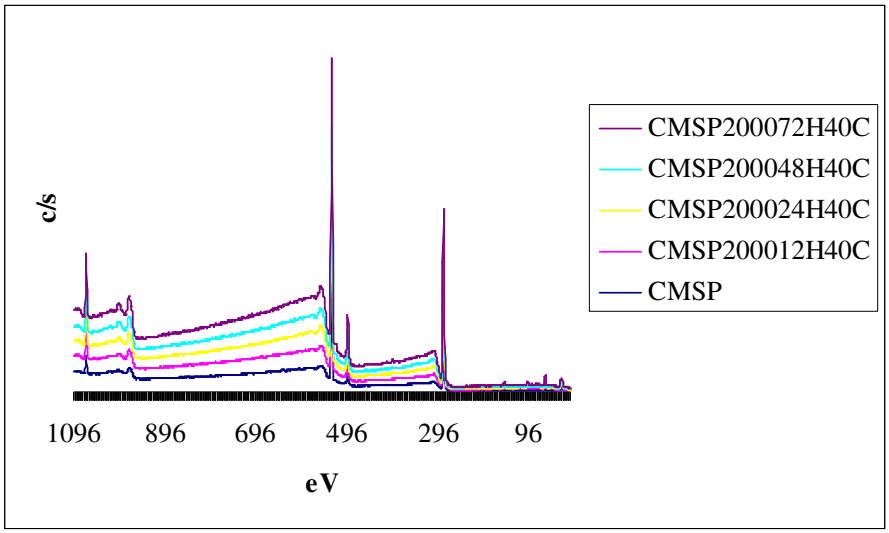


Figure B-3: Survey scans of PEG (2000 Da) grafted CM Sephadex

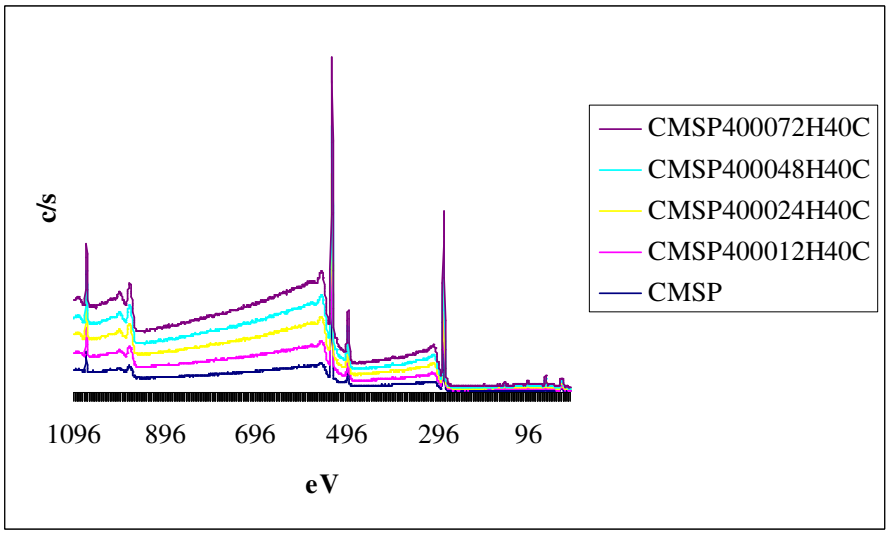


Figure B-4: Survey scans of PEG (4000 Da) grafted CM Sephadex

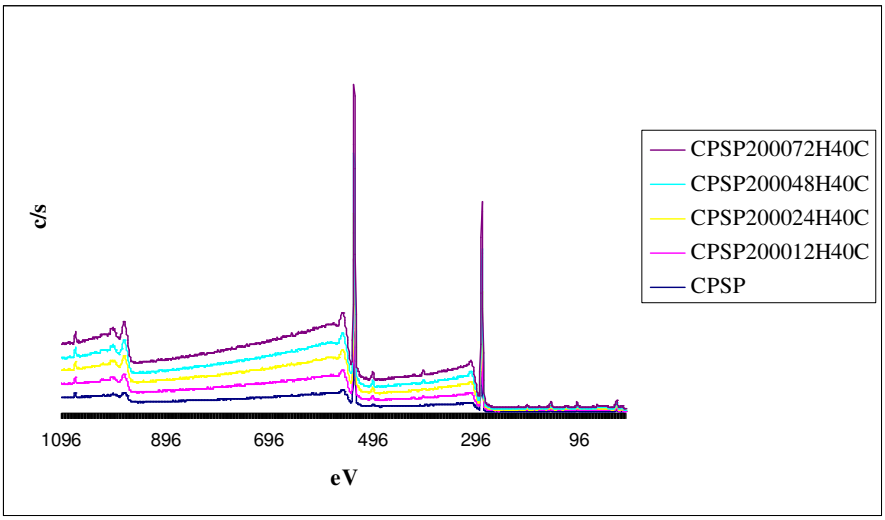


Figure B-5: Survey scans of PEG (2000 Da) grafted CP Sephadex

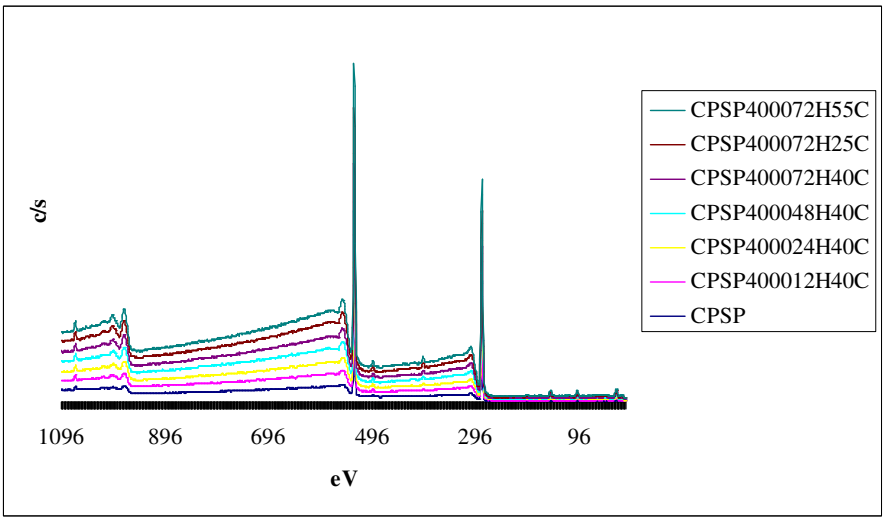


Figure B-6: Survey scans of PEG (4000 Da) grafted CP Sephadex

B.2 High resolution scans

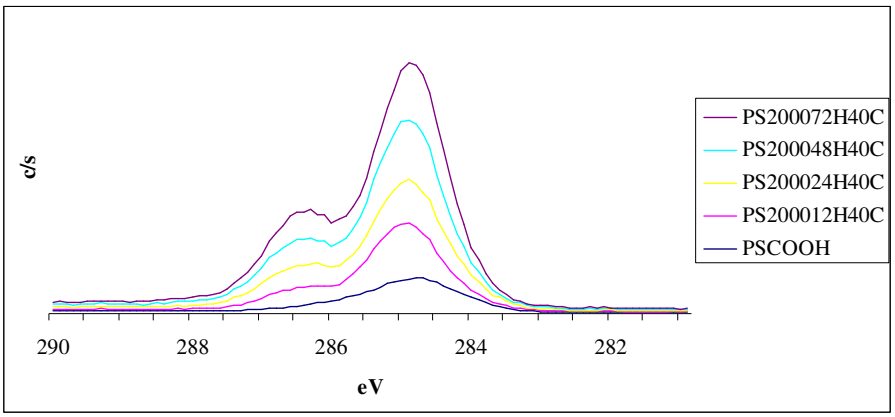


Figure B-7: HR scans of PEG (2000 Da) grafted polystyrene at 40°C

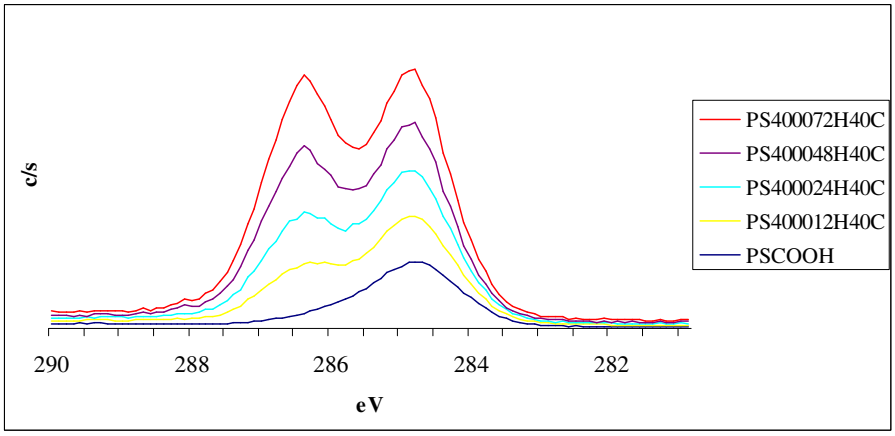


Figure B-8: HR scans of PEG (4000 Da) grafted polystyrene at 40°C

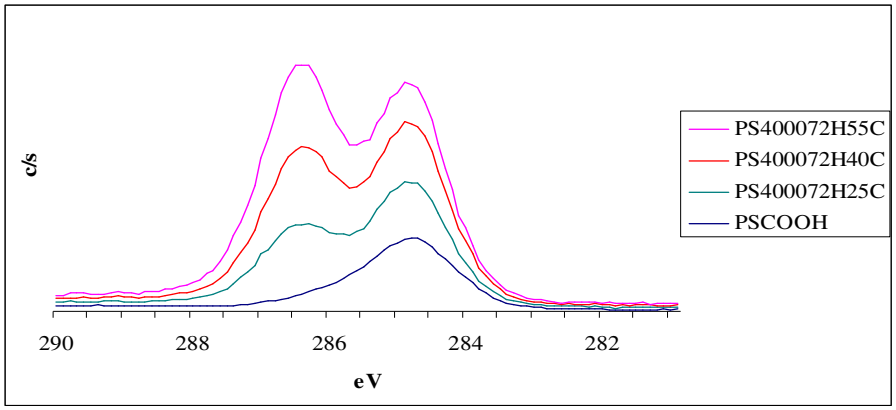


Figure B-9: HR scans of PEG (4000 Da) grafted polystyrene at various temperatures

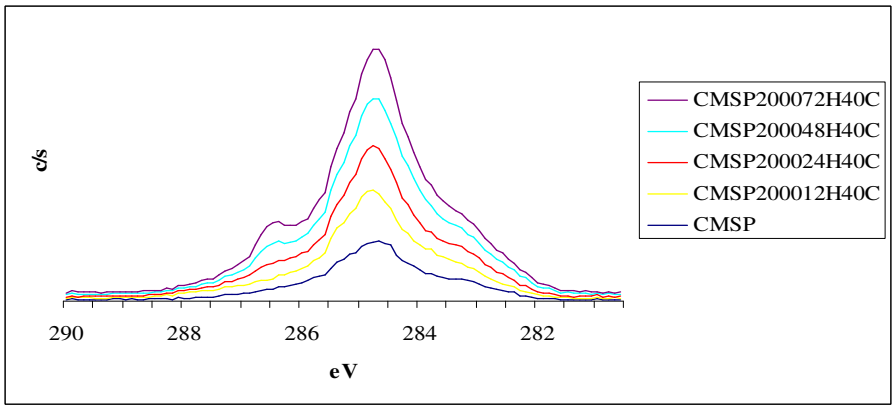


Figure B-10: HR scans of PEG (2000 Da) grafted CM Sephadex at 40°C

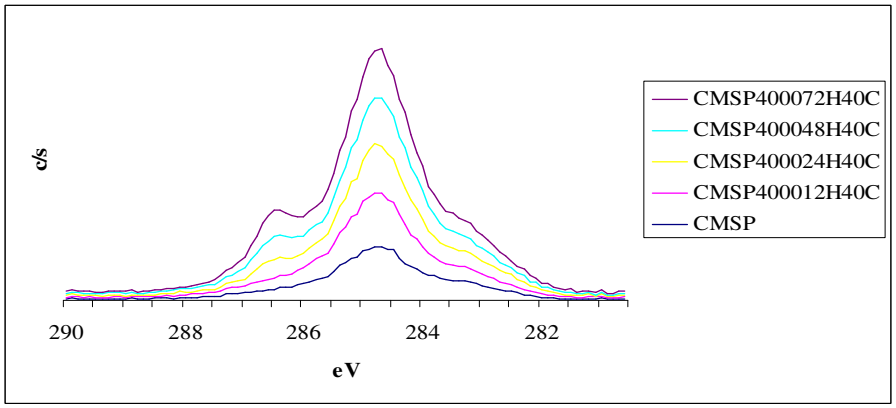


Figure B-11: HR scans of PEG (4000 Da) grafted CM Sephadex at 40°C

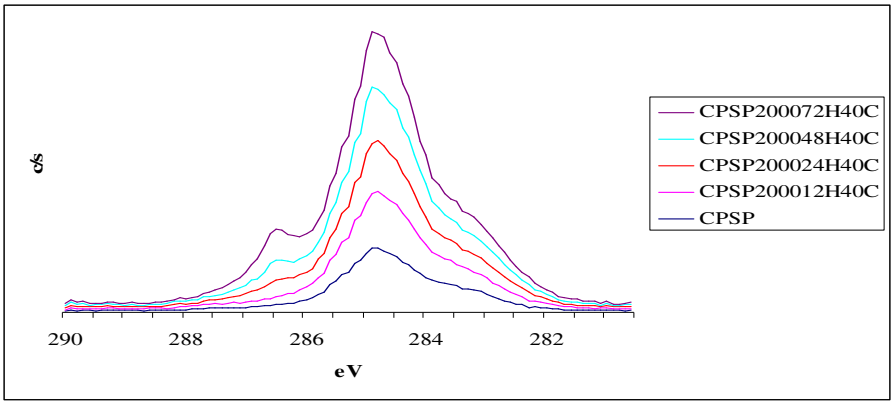


Figure B-12: HR scans of PEG (2000 Da) grafted CP Sephadex at 40°C

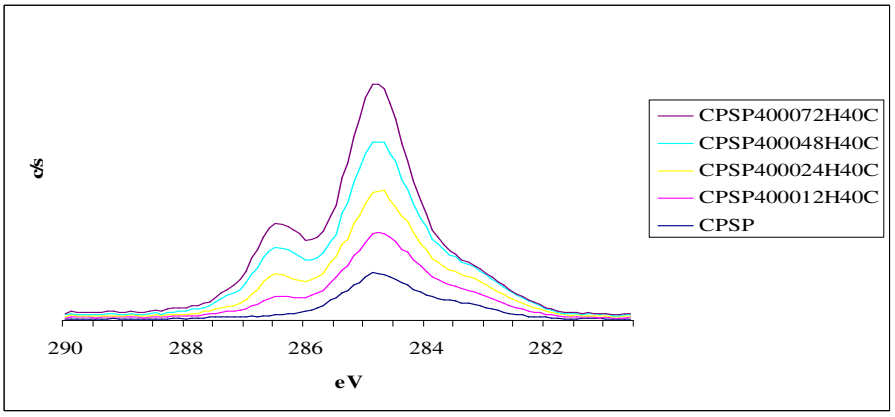


Figure B-13: HR scans of PEG (4000 Da) grafted CP Sephadex at 40°C

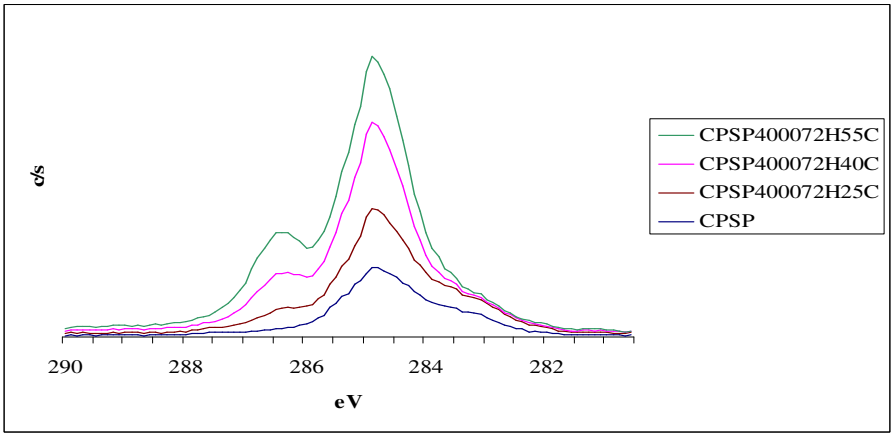


Figure B-14: HR scans of PEG (4000 Da) grafted CP Sephadex at various temperatures